

Summer 5-7-2010

Chemotaxis of amphibian pathogen *batrachochytrium dendrobatidis* in the presence and absence of antifungal metabolites produced by amphibian skin bacteria

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Chemotaxis of Amphibian Pathogen *Batrachochytrium dendrobatidis* in the Presence and
Absence of Antifungal Metabolites Produced by Amphibian Skin Bacteria

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A thesis submitted to the Graduate Faculty of

JAMES MADISON UNIVERSITY

In

Partial Fulfillment of the Requirements

for the degree of

Master of Science

Biology Department

August 2010

Acknowledgments

I would like to thank Dr. Reid Harris, my committee chair, for his guidance throughout my research as well as my committee members Dr. Grace Wyngaard, Dr. James Herrick, and Dr. Brian Walton for their valuable time. I would also like to thank Dr. Joyce Longcore who supplied me with *Batrachochytrium dendrobatidis* isolates during this research. Lab members of both Dr. Reid Harris' and Dr. Kevin Minbiole's labs need to be thanked for their time, thoughts and ideas, and previous research that has contributed to my thesis. I would also like to thank Dr. Kevin Minbiole's lab for supplying the bacterially-produced metabolites. Dr. Alex Bannigan's time and microscopy training is also acknowledged and thanked.

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Abstract

Chytridiomycosis is an amphibian skin disease that threatens amphibian biodiversity worldwide. The infectious fungal agent of chytridiomycosis, *Batrachochytrium dendrobatidis*, can affect amphibians differently in that some amphibian populations can co-exist with the fungus and others quickly succumb to disease. Amphibians in populations that co-exist with the *B. dendrobatidis* are shown to have sub-lethal infections on their skins. Several co-existing populations have also been shown to have higher proportions of individuals with antifungal skin bacteria suggesting the role of skin bacteria in disease outcome. Little is known about the mechanism(s) that antifungal skin bacteria use to ameliorate the effects of *B. dendrobatidis*. In this study I identified that a *B. dendrobatidis* isolate JEL 310 zoospores (motile infectious phase) displays chemotaxis in the presence of two bacterially-produced metabolites (2,4-diacetylphloroglucinol and indole-3-carboxaldehyde). In the presence of either metabolite, *B. dendrobatidis* (JEL 310) zoospores are more likely to move away from the metabolites. Another *B. dendrobatidis* isolate JEL 423 did not display chemotaxis in the presence of violacein (bacterially-produced metabolite). Using parameters estimated from my *B. dendrobatidis* chemotaxis study, a mathematical model illustrates that *B. dendrobatidis* JEL 310 zoospores will disperse onto the source containing no metabolite if zoospores initially start both away from the source and on the source. The model illustrates that in the presence of a bacterially-produced 2,4-DAPG, *B. dendrobatidis* JEL 310 zoospores will disperse away from the source. These results suggest that amphibians that have skin bacteria that produce 2,4-DAPG might be able to keep *B. dendrobatidis* infections below the lethal threshold and thus be able to co-exist with the fungus.

Introduction

Chytridiomycosis is an amphibian skin disease caused by the fungus *Batrachochytrium dendrobatidis* (Longcore et al. 1999). The disease causes amphibian declines and extinctions all over the world (Bosch et al. 2001, Carey et al. 2003, IUCN 2006, Rachowicz et al. 2006, Skerratt et al. 2007). Interestingly, *B. dendrobatidis* affects amphibian populations differently even within the same species. There are some amphibian populations that co-exist with *B. dendrobatidis* with sub-lethal infections and other populations (even of the same amphibian species) that decline within 1-2 years to extinction (Davidson et al. 2003, Hanselmann et al. 2004, Retallick et al. 2004).

Once a single *B. dendrobatidis* zoospore (the motile infectious phase) infects amphibian skin, *B. dendrobatidis* grows exponentially on the skin (Carey et al. 2006). Carey et al. (2006) suggest the number of *B. dendrobatidis* needed to cause mortality must surpass the lethality-threshold. Many amphibian populations that co-exist with *B. dendrobatidis* are shown to have small numbers of *B. dendrobatidis* on frog skins compared to populations to go extinct. Low numbers of *B. dendrobatidis* on frog skins results in sub-lethal infections. One explanation for this may be differences in amphibian defenses present in populations such as amphibian skin bacteria.

Many bacterial species can be isolated from amphibian skins (Becker et al. 2009, Harris et al. 2006, Harris et al. 2009a, Harris et al. 2009b, Lam et al. 2010, Lauer et al. 2007, Lauer et al. 2008, Woodhams et al. 2007a, Woodhams et al. 2007b). Some of these bacteria produce antifungal metabolites that are inhibitory to *B. dendrobatidis*. There are three hypothesized mechanisms that amphibian skin bacteria use to reduce the number of *B. dendrobatidis* zoospores (its motile pathogenic phase): 1) skin bacteria may reduce the

number of available attachment sites on amphibian skin, 2) antifungal metabolites produced by skin bacteria may directly kill *B. dendrobatidis*, and 3) skin bacteria may alter *B. dendrobatidis* zoospore behavior resulting in chemotaxis away from bacterially-produced antifungal metabolites. For my thesis research, I have chosen to focus on the third hypothesis.

The objective of this study was to investigate *B. dendrobatidis* behavior response to antifungal metabolites produced by skin bacteria through its movement. Disease transmission may be a function of behavior movement response of *B. dendrobatidis*. Measuring the response of the pathogen in the presence of different concentrations of antifungal metabolites may allow an improved understanding of the transmission (infection) and re-infection of *B. dendrobatidis*. From these measurements I estimated parameters for a mathematical model used to illustrate how fast a *B. dendrobatidis* zoospore will reach an amphibian with bacterially-produced metabolites on its skin. I also used these estimated parameters to illustrate how zoospores will disperse onto an amphibian with metabolites present on its skin. Below, I will briefly review emerging infectious diseases, amphibian declines, chytridiomycosis, and published research about the amphibian innate immune system that protects against the pathogen.

Emerging Infectious Disease (EIDs)

Over the past two decades there have been a number of emerging infectious diseases (EIDs), both human and wildlife, that are of concern, and the frequency of emergence of novel EIDs is increasing (Acevedo-Whitehouse and Duffus 2009, Dazak et al. 2000). The increased frequency of the emergence of novel EIDs is believed to be a

result of changes in the environment and human encroachment. Jones et al. (2008) analyzed the origins of 335 EIDs between the years 1940 and 2004. They found that the origins of EIDs are significantly correlated with socio-economic, environmental, and ecological factors. For example, coral bleaching has increased dramatically during the last few decades. The incidences of coral bleaching are highly correlated with increased sea-water temperature. At high summer sea-water temperatures, *Vibrio shiloi* expresses virulence genes for adhesion. These genes allow the bacterium to displace the normal microbiota, adhere to the coral mucus, and penetrate the coral epidermis where it produces toxins that lead to disease (Rosenberg and Ben-Haim 2002).

Emerging infectious diseases pose a threat to global biodiversity. Daszak et al. (2000) argue that EIDs threaten biodiversity by depopulating host populations and causing chronic population depression. The geographic spread of chytridiomycosis, an EID plaguing amphibians worldwide, greatly threatens amphibian biodiversity. Its global distribution and high virulence as well as other factors influencing amphibian declines are real concerns for amphibian conservation.

Amphibian Crisis

It has been estimated that at least a third of amphibians worldwide are threatened with extinction (Briggs et al. 2005, Lips et al. 2008, Moore and Church 2008, Rachowicz et al. 2006, Stuart et al. 2004) (Figure 1). Amphibian declines and extinction are only a part of what is being called the sixth mass extinction, which includes other vertebrate and invertebrate classes (Wake and Vredenburg 2008). The cause of amphibian declines is hypothesized to be due to a synergy of habitat destruction, exotic predators, competition

with non-native species, pollution, utilization as food and pets (Blaustein and Wake 1990, Fisher and Garner 2007, Stuart et al. 2004), effects of climate change (Lips et al. 2008, Pounds and Crump 1994), increased levels of ultraviolet radiation (Blaustein et al. 1995), and disease (Carey and Alexander 2003; Pounds and Crump 1994; Pounds et al. 2006).

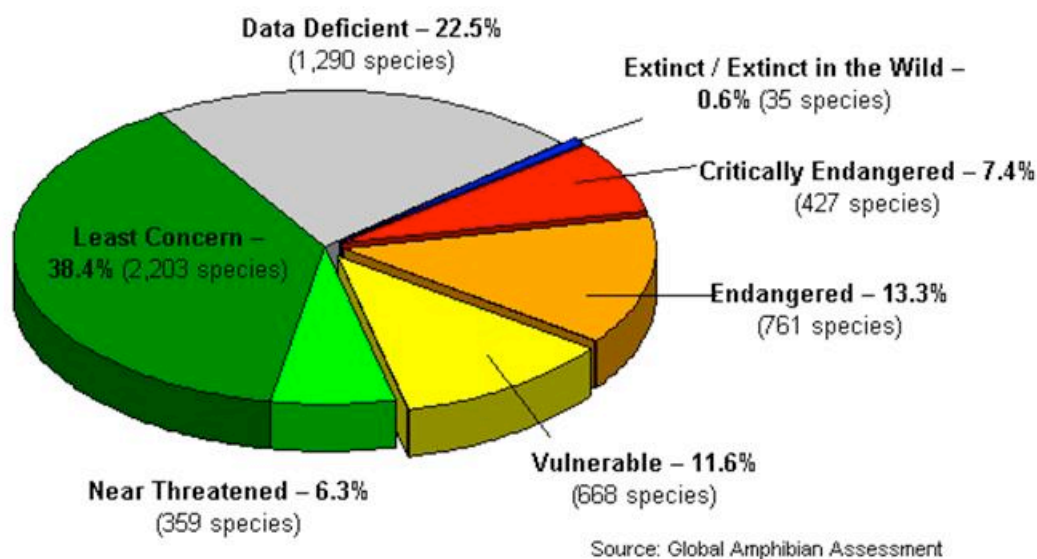


Figure 1. Global amphibian assessment published in 2004 that shows the percentage of amphibian species vulnerable to extinction. It is estimated that a third of amphibians are vulnerable, but, if data deficient species are taken into account, the estimate increases to nearly 40-50% of amphibians worldwide. (Stuart et al., 2004).

Although it is argued that deforestation and habitat loss are the major factors in amphibian declines, Stuart et al. (2004) listed 207 amphibian declines that they reported as “enigmatic”. These amphibian declines were reported in pristine areas such as natural parks and areas unaffected by deforestation. Skerratt et al. (2007) demonstrated that the presence of an EID, chytridiomycosis, is the primary cause of these enigmatic declines.

Chytridiomycosis has contributed to declines in pristine areas and is responsible for amphibian extinctions on all continents where amphibians are found (Bosch et al. 2001, Carey et al. 2003, IUCN 2006, Rachowicz et al. 2006, Skerratt et al. 2007). The major factors of amphibian declines such as habitat loss and now the emergence of chytridiomycosis in areas unaffected by habitat loss leave amphibians extremely susceptible to extinction.

Chytridiomycosis

Chytridiomycosis was first documented in 1998 as the cause of declines of amphibians in Australia and Central America (Berger et al. 1999). In 1999, Longcore et al. reported that the agent of chytridiomycosis is a fungus which they named *Batrachochytrium dendrobatidis*, a zoosporic chytrid fungus that is the only known chytrid to parasitise vertebrates; however there are many other species of chytrid that parasitise plants (Sharma 2004). This pathogen has dimorphic life history: (1) a motile zoospore stage (the infective phase) and (2) a stationary zoosporangium stage (growing phase) (Berger et al. 2005a). The fungus has been documented to expand its range as an advancing wave (Berger et al. 1999, Lips et al. 2005, Lips et al. 2008, Moore and Church 2008).

The emergence of *B. dendrobatidis* is not well understood and the two opposing hypotheses that attempt to explain the emergence of this amphibian disease are the novel pathogen hypothesis and the endemic pathogen hypothesis (Carey and Alexander 2003, Pounds and Crump 1994, Pounds et al. 2006, Kilpatrick et al. 2010). In support of the novel pathogen hypothesis is the genetic similarity between isolates of *B. dendrobatidis*

found worldwide (James et al. 2009, Morehouse et al. 2003). The little variation found among isolates from different continents suggest that *B. dendrobatidis* has been introduced from a source population into different areas of the world and spread rapidly throughout. Support for the endemic pathogen hypothesis would entail that the levels of genetic variation observed from isolates would show more genetic divergence. There is some evidence that chytridiomycosis outbreaks are correlated with increased temperatures and thus may contribute to the pathogen becoming endemic. This evidence provides some support for the endemic pathogen hypothesis, which states that *B. dendrobatidis* has been historically endemic and conditions are changing to favor its pathogenesis. Pounds et al. (2006) correlated outbreaks of chytridiomycosis and global warming by analyzing the time at which an outbreak occurred and changes in sea surface and air temperatures. Changes in global temperature may act as a stress mechanism for amphibians and cause changes in the immune system, making amphibians more susceptible to disease (Davidson et al. 2007, Raffel et al. 2006) or these changes may favor the life-cycle of *B. dendrobatidis*. It is possible the *B. dendrobatidis* has been recently introduced into various parts of the world and that changing climatic conditions facilitates its emergence. Thus, both hypotheses may be correct to some degree.

The infective stage of *B. dendrobatidis* attacks the keratinized cutaneous layer of post-metamorphic amphibians and the keratinized jaw sheaths and tooth rows of larvae (Rachowicz and Vredenburg 2004, Rachowicz et al. 2006). Infection occurs most often on the ventral surface of the amphibian, but severe infections can spread over the entire body of the amphibian (Berger et al. 2005a, Pessier et al. 1999). During infection, hyperkeratosis (an overproduction of keratin in the epidermis) is observed and, in deeper

regions of the epidermis, hyperplasia (abnormal proliferation of cells) is common and the epidermis will begin to slough (Berger et al. 1998, Berger et al. 2005a, Longcore et al. 1999). Behavioral changes are often observed in infected individuals such as anorexia, lethargy, unresponsiveness to stimuli, the inability of the amphibian to right itself (Berger et al. 2005b, Nichols et al. 2001), and infected individuals are sometimes observed trying to dry themselves by lifting their limbs into the air or moving away from water sources (Becker and Harris 2010).

Little is understood about the mode of mortality chytridiomycosis causes, but it is hypothesized that *B. dendrobatidis* causes death in two ways: (1) *B. dendrobatidis* may release a deadly toxin that diffuses into the blood stream (although to date, none have of been found), and (2) *B. dendrobatidis* disrupts osmotic and ionic balances of the amphibian due to hyperkeratosis and hyperplasia (Berger et al. 1998, Berger et al. 2005a, Carey et al. 2003, Voyles et al. 2007, Voyles et al. 2009). Disruption of osmotic and ionic balances has been documented to cause cardiac distress and failure in post-metamorphic amphibians (Voyles et al. 2009). Mortality is documented primarily in post-metamorphic amphibians, but can also occur in larvae (Blaustein et al. 2005).

A single zoospore has the capability of establishing infection due to exponential growth of the pathogen observed on amphibian skin. Carey et al. (2006) show that one zoospore was able to cause morbidity and mortality in the boreal toad, *Bufo boreas*. When a single zoospore comes in contact with an amphibian it begins to form a zoosporangium and produces more zoospores within the zoosporangium that are later released. These released zoospores have the capability to re-infect the amphibian or be transmitted to a new host (Rachowicz et al. 2006). Carey et al. (2006) also demonstrated

that *B. dendrobatidis* zoospore numbers on toad skins had to surpass a threshold of 10^7 - 10^8 zoosporangia per toad in order to cause mortality of *B. boreas*.

Moss et al. (2008) recently found that during the motile zoospore stage, *B. dendrobatidis* displays chemotactic ability to respond with positive movement towards hydrolyzed casein (known to support *B. dendrobatidis* growth), gelatin hydrolysate (a constituent of the culture media used for *B. dendrobatidis*), and cysteine (an abundant amino acid of keratin found in epidermal cells of amphibian skin). Their findings suggest that the pathogen is capable of responding to keratin in amphibian skin and can use chemotaxis as a method to detect the amphibian host. Once a single zoospore detects and comes in contact with a susceptible amphibian host, exponential growth can quickly surpass a lethality-threshold resulting in mortality.

What is very intriguing about this disease is that not all amphibian species decline to extinction and some species are able to co-exist with the fungal pathogen (Davidson et al. 2003, Hanselmann et al. 2004, Retallick et al. 2004). Even more intriguing is that the same is true within some amphibian species, where some populations are able to co-exist and other populations of the same species drastically decline to extinction. For example, *Rana sierrae*, the mountain yellow-legged frog of the Sierra Nevada Mountains in California, USA has some populations of *R. sierrae* that have been co-existing with *B. dendrobatidis* for >6 years and other populations that go extinct within 1-2 years after the pathogen first emerges (Lam et al. 2010, Rollin-Smith et al. 2006, Woodhams et al. 2007b). A better understanding of differences between populations that co-exist with the pathogen and populations that go extinct due to the pathogen such as differences in amphibian defenses is strongly needed. Differences in amphibian defenses may lead to

insights into finding a more environmentally friendly way to stop the spread of the disease and allow for the re-introduction of captive frogs from survival assurance colonies.

Amphibian Defenses Against Chytridiomycosis

There is considerable variation in amphibian species' and populations' response to chytridiomycosis (Lips et al. 2005); this may be due to variation in the virulence of *B. dendrobatidis* isolates (Berger et al. 2005b, Retallick and Miera 2007), although preliminary data show that there is little genomic variation among *B. dendrobatidis* isolates sampled (James et al. 2009, Morehouse et al. 2003). Another source of variation may be differences in the types and magnitude of immune defenses the amphibian is able to mount against the pathogen.

There are two major components of the immune system: (1) the adaptive immune system and (2) the innate immune system. Although it has been found that amphibians do elicit an adaptive immune response to *B. dendrobatidis* by producing antibodies, there is no published evidence that this response produces protection or immunity (Berger et al. 2002). A possible explanation for the inadequacy of the adaptive immune response is that the response is "too little, too late". The response does not occur in time to prevent the mortality from the disease. Another possibility is that the antibodies are inadequately delivered to the infection due to thickened epidermis. A third possibility is that *B. dendrobatidis* is turning off the amphibians' immune system. Recent work shows that infected amphibians' adaptive immune system loci are either not up regulated or are down regulated (Rosenblum et al. 2009).

The innate immune system is the first line of defense against *B. dendrobatidis* and has several components that are capable of providing protection for the amphibian. There are three known components of the innate immune system that may give amphibians protection from *B. dendrobatidis*: (1) the epidermis as a barrier, (2) antimicrobial peptides the amphibian produces, and (3) antifungal symbiotic bacteria that lives on amphibian skin (although some researchers may be uncomfortable calling symbiotic bacteria part of the innate immune system).

Research to investigate how the epidermis as a barrier may protect against chytridiomycosis has not been explored. It is possible that amphibians with thick epidermal layers may have better protection against the pathogen. Also, variation in the components of mucus secretions and differences in the thickness of the mucus layer may also provide explanations for the varying degree of susceptibility in different amphibian species to the *B. dendrobatidis*. Mucus can protect amphibians from both bacterial and fungal pathogens (Fontana et al. 2006). Fontana et al. (2006) describe a new gland named mucus-producing granular glands (MPGGs) that produce mucus in specialized granular glands. MPGGs produce acidic mucus relative to neutral mucus produced in mucous glands. They hypothesize that neutral and acidic mucus may form two layers of mucus that consists of a thin, watery neutral mucus layer with a thicker acidic layer underneath. More research would be necessary to understand how the epidermis as a barrier and the mucus layer are involved in the susceptibility of amphibians to chytridiomycosis.

Antimicrobial peptides (AMPs) are the best studied of the three components of the amphibian innate immune system (Davidson et al. 2007, Rollins-Smith et al. 2002,

Woodhams et al. 2007a). These peptides are produced and stored in granular glands in the dermis of amphibian skin and found predominately on the dorsal surface of the amphibian. They are released in response to stimulation from the sympathetic nervous system (Mills and Prum 1984). AMPs have been documented as having inhibitory activity against bacteria, viruses, including HIV (VanCompernelle et al. 2005), protozoa, and fungi (Rinaldi 2002, Zasloff 2002). Many of these peptides have shown inhibitory activity *in vitro* against *B. dendrobatidis* (Davidson et al. 2007, Rollins-Smith et al. 2002, Rollins-Smith et al. 2005, Rollins-Smith et al. 2006). Amiche et al. (1999) suggest that each species has a unique set of AMPs. Among individuals, these peptides may vary in the density and concentration in which they occur on amphibian skin and the variety of AMPs present at any given time (Tennessen et al. 2009). Woodhams et al. (2007a) performed laboratory experiments with four Australian amphibian species in which survival rate *in vivo* and, in a separate experiment, the *in vitro* effectiveness of the AMPs produced by each species were studied. They experimentally infected the frogs with the pathogen and observed survival rate. The more effective the species' AMPs were against *B. dendrobatidis in vitro*, the higher the species' survival rate. However, factors such as environmental change, pesticides, and continual exposure to the pathogen may minimize the effectiveness of AMPs produced by amphibians (Davidson et al. 2007, Rollins-Smith et al. 2002, Rollins-Smith et al. 2006). These factors may influence the production of AMPs as well their depletion. For example, pesticides may block chemical signals that result in the amphibian's capabilities to produce a particular AMP although no evidence of this exists in the literature.

A less studied component of the innate immune system is the symbiotic bacteria that reside on amphibian skin. Amphibian skin provides a seemingly ideal habitat for microorganism colonization in that it is moist and nutrient-rich (Guirard and Snell 1962). Bettin and Greven (1986) demonstrated that amphibian skin is colonized by bacteria and by their presence suggest that these bacteria are somewhat resistant to the antimicrobial peptides the amphibian produced. Their discovery suggests that some species of bacteria may have co-evolved to be resistant to the AMPs found on the amphibian species they inhabit.

Relationships between commensal bacteria and eukaryotic organisms often occur in nature and often these bacteria protect their hosts from pathogens. Symbiotic bacteria that protect organisms from fungi have been identified in Caribbean shrimp, *Palaemon macrodactylus* (Gil-Turnes et al. 1989), pea aphids, *Acyrtosiphon pisum* (Scarborough et al. 2005), the four-toed salamander, *Hemidactylium scutatum* (Banning et al. 2008, Lauer et al. 2008), the eastern red back salamander, *Plethodon cinereus* (Becker et al. 2009, Harris et al. 2006, Harris et al. 2009b, Lauer et al. 2007, Lauer et al. 2008) and the mountain yellow-legged frog, *Rana sierrae* (Lam et al. 2010, Harris et al. 2009a, Woodhams 2007b).

In 2006, Harris et al. identified 10 genera of bacteria isolated from the skins of the eastern red-backed salamander, *Plethodon cinereus*, and the four-toed salamander, *Hemidactylium scutatum*, that displayed antifungal activity against *B. dendrobatidis* *in vitro*. They first isolated bacteria from the salamander skins and then challenged the bacteria against *B. dendrobatidis* *in vitro*. They discovered that some of the bacteria produced a zone of inhibition around the pathogen suggesting antifungal activity.

More recently, it has been found in field surveys that populations of *Rana sierrae* that co-exist with *B. dendrobatidis* have higher proportions of individuals with anti-*B. dendrobatidis* skin bacteria than populations that are found to go extinct due to the pathogen (Lam et al. 2010, Woodhams et al. 2007b). In the initial field study, Woodhams et al. (2007b) show that a population where *B. dendrobatidis* had recently emerged and that was predicted to go extinct (due to the history of populations in the same area) had a low proportion of individuals with at least one anti-*B. dendrobatidis* skin bacterial species relative to another population persisting with *B. dendrobatidis*. The population with a low proportion of individuals with protective skin bacteria went extinct the following year. An extension of the Woodhams et al. (2007b) field study shows that a *R. sierrae* population with a higher proportion of individuals with anti-*B. dendrobatidis* bacteria survived the arrival of *B. dendrobatidis* even though it was in an area where historically all populations declined to extinction after the emergence of the pathogen. Lam et al. (2010) performed a field survey on a *R. sierrae* population before the emergence of the pathogen. This population was predicted to go extinct due to historical field surveys where all populations in the same geographical field location became extinct 1-2 years after the arrival of *B. dendrobatidis* (Vance Vredenburg, personal communication). The pathogen emerged in this population a year after the initial field survey, and two years later the population is still found to be surviving and endemic with *B. dendrobatidis*. These field surveys suggest a threshold proportion of individuals need anti-*B. dendrobatidis* bacteria in order for the population to co-exist with the fungus (Figure 2). This is analogous to herd immunity in which only a proportion of the herd or population needs to be immunized in order to establish protection from a pathogen for the

entire population (Gonclaves 2008). These studies suggest that approximately 80% of individuals need to have anti-*B. dendrobatidis* protective skin bacteria for co-existence with *B. dendrobatidis* to occur and suggests that the basic reproductive number, R_0 , of *B. dendrobatidis* is less than 5 (Lam et al. 2010). These studies suggest that future bioaugmentation treatment would only need to include a proportion of the population instead of the whole population.

These field studies are consistent with the hypothesis that populations with higher proportions of individuals with anti-*B. dendrobatidis* bacteria can co-exist with the fungus. Although the survival of populations with a high percentage of individuals with anti-*B. dendrobatidis* does not prove the hypothesis, it does strengthen it and indicates that more research is merited. Another interesting result from these *R. sierrae* field surveys is that the populations that co-exist do so with a significantly lower density of *B. dendrobatidis* zoospores on their skins than the populations that went extinct (Woodhams et al. 2007b) (Figure 3). This also suggests that there may be a threshold of zoospores needed to cause mortality of the amphibian (Carey et al. 2006) and that populations that co-exist with *B. dendrobatidis* do so with sub-lethal infections. The higher proportion of individuals with anti-*B. dendrobatidis* bacteria correlated with individuals that have sub-lethal infections suggests that skin bacteria plays a role in driving *B. dendrobatidis* densities down and below lethality-threshold.

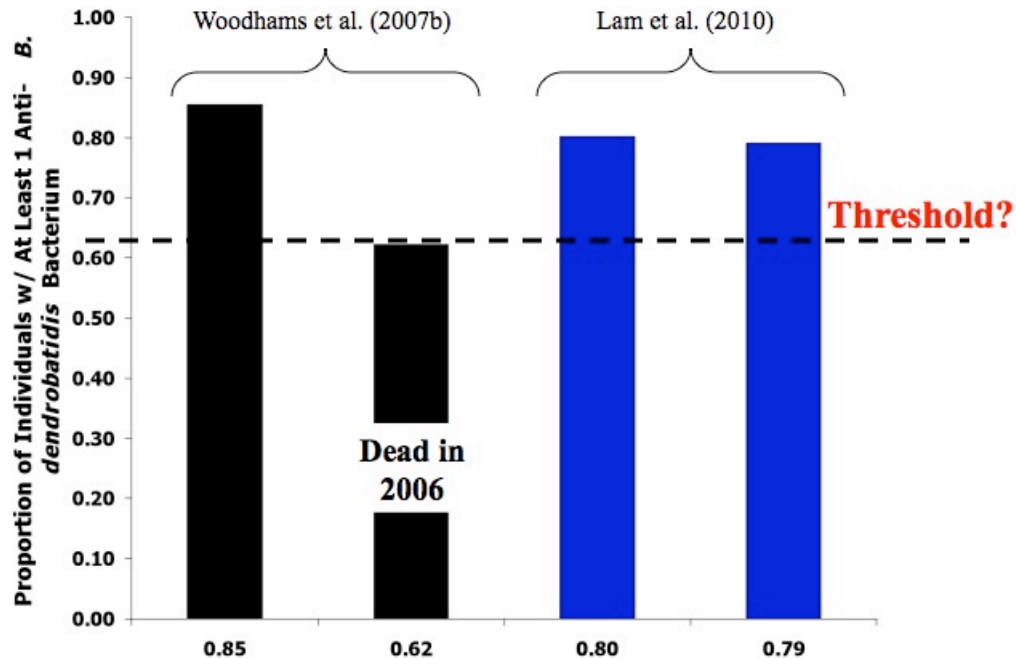


Figure 2. This graph is a compiles data from Woodhams et al. (2007b) and Lam et al. (2010). The dotted line is a theoretical threshold line where all populations that have proportions of individuals with anti-*B. dendrobatidis* bacteria above the threshold are co-existing with the fungus.

Several anti-*B. dendrobatidis* bacterial isolates have been found to produce antifungal metabolites that are capable of inhibiting *B. dendrobatidis* *in vitro* (Brucker et al. 2008a, Brucker et al. 2008b). Harris et al. (2009b) show that the addition of *Pseudomonas reactants*, a previously identified bacterium found to inhibit *B. dendrobatidis*, to the eastern red-backed salamander, *Plethodon cinereus*, ameliorated the effects of chytridiomycosis. The salamanders that were bathed with and colonized by *P. reactants* lost less weight, a symptom of chytridiomycosis, than salamanders exposed only to *B. dendrobatidis*. Harris et al. (2009a) also show that the addition of *Janthinobacterium lividum*, another bacterium found to be anti-*B. dendrobatidis*, to *Rana sierrae* juveniles prevented the colonization of *B. dendrobatidis* and had lasting positive

effects for 140 days. The frogs that were bathed in and colonized with *J. lividum* were also found to have violacein, an antifungal metabolite produced by *J. lividum* (Brucker et al. 2008b), on their skins, suggesting that bio-augmentation of *J. lividum* increased violacein concentration on the skins of *R. sierrae*. These studies, in conjunction with field surveys, strongly suggest that bio-augmenting a population to have a higher proportion of individuals with anti-*B. dendrobatidis* skin bacteria may be a feasible tool to controlling the pathogen in wildlife. If there is a threshold proportion of individuals needed to protect the entire population, not all of the individuals would need to be treated (this is analogous to herd-immunity).

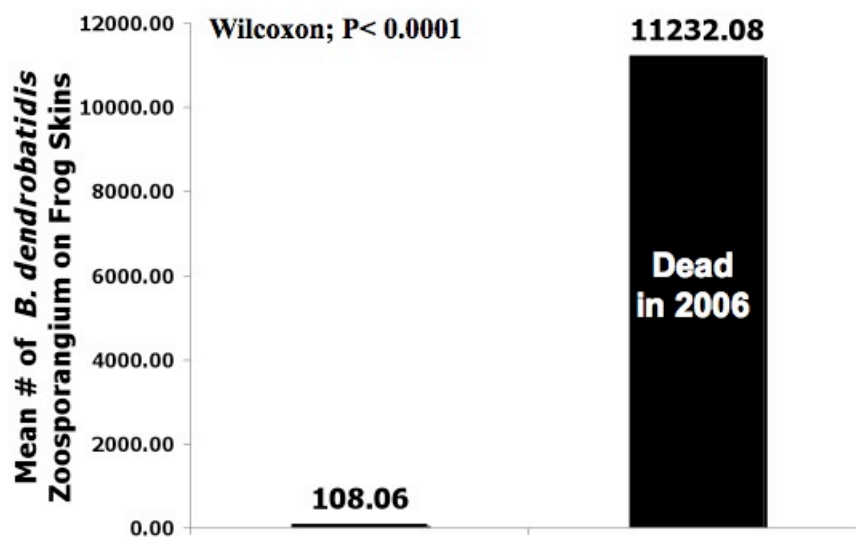


Figure 3. Zoosporangium on frog skins data from Woodhams et al. (2007b). The co-existing population had a significantly larger proportion of individuals with anti-*B. dendrobatidis* bacteria on frog skins (Fisher's Exact Test; $P = 0.045$).

Understanding the Relationship Between B. dendrobatidis and the Innate Immune System by Modeling

The conservation implications for using anti-*B. dendrobatidis* bacteria in nature need to be further researched. For example, interactions of symbiotic bacteria and AMPs are likely to influence the success of the application of anti-*B. dendrobatidis* skin bacteria to an amphibian population that may result in the protection from the pathogen. However, field studies and experiments to study the interactions of these components of the innate immune system and the pathogen may be complicated and costly to study *in vivo*. There are many uncontrollable interactions that may complicate designing a plan to conserve amphibian species. Mathematically modeling this dynamic system is one feasible method to further studying this system.

Two recent mathematical models of *B. dendrobatidis* and its host have been unable to predict co-existence between amphibians and *B. dendrobatidis* despite using a variety of parameter values (Briggs et al. 2005, Mitchell 2008). The Briggs et al. (2005) model could not explain the differences between populations of *R. sierrae* co-existing with *B. dendrobatidis* and those that declined to extinction by differences in transmission rate of *B. dendrobatidis*, susceptibility, infectiousness, or population size. Their model only predicted one of two outcomes; there was either extinction of the amphibian host or extinction of the pathogen. One criticism of this model and possible explanation for the models inability to predict co-existence may be the lack of a re-infection parameter, which is the proportion of *B. dendrobatidis* zoospores that re-infects its host. The model proposed by Mitchell et al. (2008) model could only predict the co-existence of the toad, *Bufo bufo*, if the model incorporated saprobic reproduction of *B. dendrobatidis* (which

has not been observed) for a few *B. bufo* generations. However, eventually *B. bufo* went extinct as *B. dendrobatidis* density increased. This model also does not include a re-infection parameter, but assumes that all juvenile toads that were infected with the fungus left the population and died.

A re-infection parameter of the amphibian host is needed to reflect the biology of *B. dendrobatidis*. As mentioned earlier, *B. dendrobatidis* has the capability to re-infect its host and, thus, causes exponential growth of zoospores on amphibian skin. However, Woodhams et al. (2007b) found that populations of *R. sierrae* that were co-existing with *B. dendrobatidis* had significantly lower averages of zoospores on frog skins and that these populations had higher proportions of individuals with anti-*B. dendrobatidis* skin bacteria. This suggests that the role of amphibian defenses is to lower the re-infection parameter of *B. dendrobatidis* and control the growth of zoospores on amphibian skins which may result in co-existence of the amphibian and fungus.

Study System

For my thesis I have investigated the motile zoospore life-stage of *B. dendrobatidis* in the presence and absence of metabolites produced by known amphibian skin bacteria to further investigate the role of these metabolites in transmission (infection) and re-infection. As proposed by Moss et al (2008), *B. dendrobatidis* possesses chemotaxis in the presence of constituents of its food source. I have expanded this idea to investigate if *B. dendrobatidis* is also chemotactic in the presence of metabolites that display anti-*B. dendrobatidis* properties. Although known concentrations of bacterially-produced metabolites on amphibian skin are poorly estimated due to small sample size and few

species studied, it is hypothesized that concentrations are most likely variable and unevenly dispersed on amphibian skin. This is due to the idea that some microenvironments (Figure 4A-D) on amphibian skin would attract high concentrations of bacterial growth (such as mucus glands) and other microenvironments would attract low concentrations of bacterial growth (such as granular glands that produce AMPs active against bacteria).

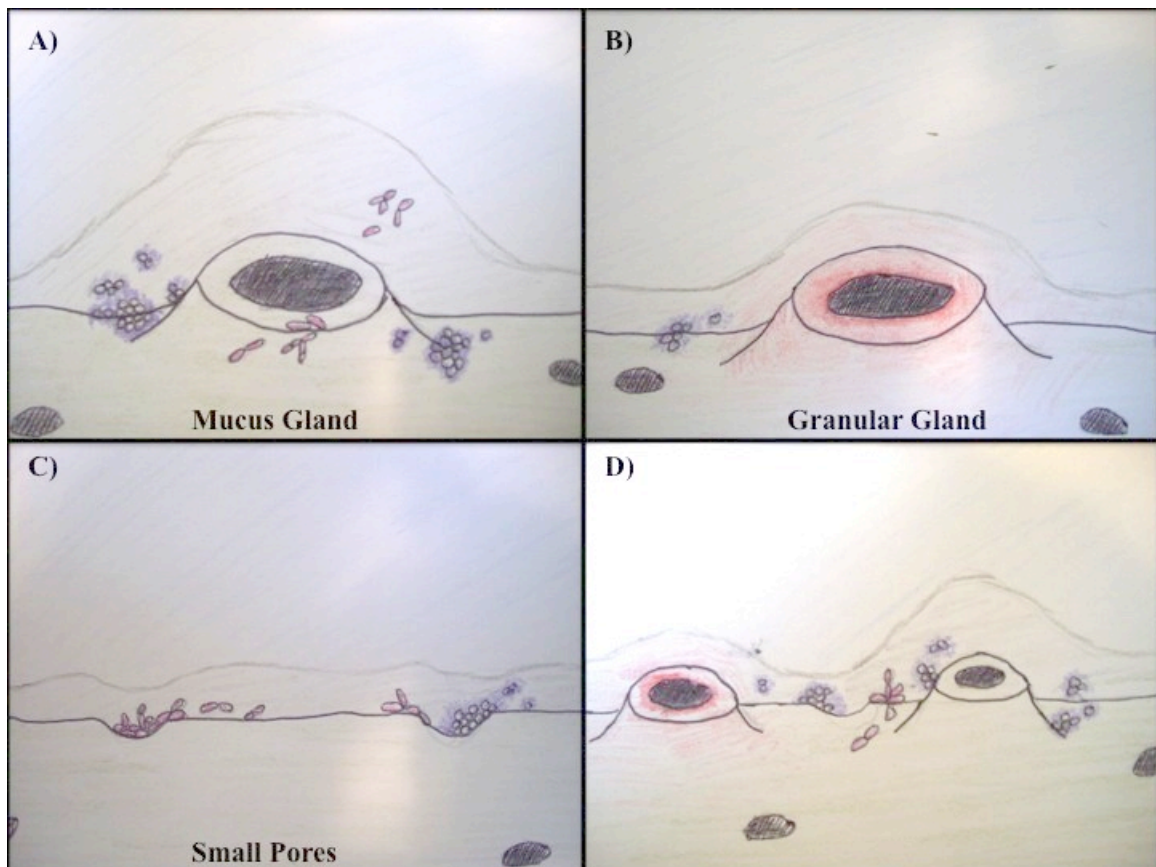


Figure 4. Three different types of microenvironments (A-C) found on amphibian skin that would determine different amounts and composition of bacterial growth. The mucus gland (A) is surrounded by a thicker mucus layer and has several different bacteria species surrounding the gland. The granular gland (B) is distinguished from the mucus gland by a gradient of AMPs (shown in red) and would only attract bacteria species that are AMP tolerant. Small pores (C) along the epidermis would attract different bacteria and possibly create anaerobic environments. Bacteria are shown to inhabit both the surface layer of the epidermis as well as in the mucus layer itself. An illustration with all three microenvironments is shown by D.

To study chemotaxis of *B. dendrobatidis* zoospores, I followed individual zoospore movement away and towards metabolites produced by amphibian skin bacteria.

Metabolites Produced by Anti-B. dendrobatidis Skin Bacteria

I investigated three metabolites produced by known amphibian skin bacteria that show inhibitory activity against *B. dendrobatidis*. These three metabolites were chosen based on their known anti-*B. dendrobatidis* properties and availability. These metabolites have all been identified from skin bacteria isolated from amphibian skins.

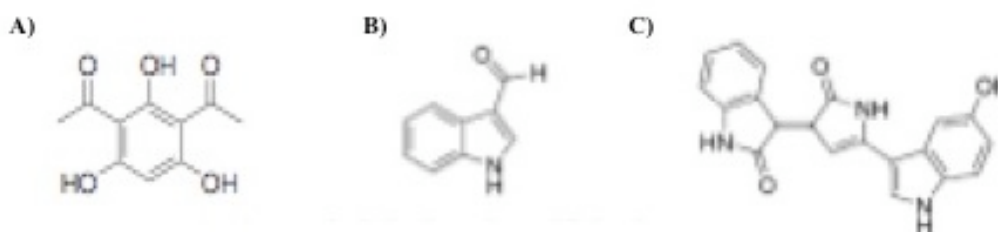


Figure 5. Chemical structures of bacterially-produced metabolites found on amphibian skins. (A) 2,4-diacetylphloroglucinol, (B) indole-3-carboxaldehyde, (C) violacein

The metabolite 2,4-diacetylphloroglucinol (2,4-DAPG) has antiviral, antibacterial, antifungal, antihelminthic, and phytotoxic properties (Weller et al. 2007) (Figure 5). This metabolite has been identified in several species of *Pseudomonas* and is a known protector against many root and seedling diseases (Weller et al. 2007). It was first shown to have anti-*B. dendrobatidis* properties when identified from *Lysobacter gummosus* isolated from the skins of amphibians (Brucker et al. 2008a). The anti-*B. dendrobatidis* bacterium has been identified on the eastern red-backed salamander, *Plethodon cinereus*, and the four-toed salamander, *Hemidactylium scutatum* (Harris et al. 2006). Using a 96-well inhibitory growth assay, Brucker et al. (2008a) determined that 2,4-DAPG has

inhibitory properties against *B. dendrobatidis* ($IC_{50}=8.73\mu M$, $MIC=136.13\mu M$) although the mechanisms of this inhibition are unknown.

Two other anti-*B. dendrobatidis* metabolites identified from amphibian skin bacteria used during this experiment are indole-3-carboxaldehyde (I3C) and violacein (Figure 5). These metabolites were identified from the bacterium *Janthinobacterium lividum* (Brucker et al. 2008b) that has been cultured from the skins of the salamander *P. cinereus* (Harris et al. 2006) and from the skins of the frogs *Rana muscosa* (Woodhams et al. 2007b) and *Rana sierrae* (Lam et al. 2010). The metabolite I3C is more commonly known as a hydrolysis product found in the plant family *Cruciferae* and for its anti-tumorigenesis properties (Anderton et al. 2004, Chohan et al. 2010), but has also been identified in several bacteria species (Brucker et al. 2008b, Gutierrez-Lugo et al. 2005). I3C was also shown to be inhibitory against *B. dendrobatidis* using 96-well inhibition assays ($IC_{50}=0.13\mu M$; $MIC=68.9\mu M$) (Brucker et al. 2008b). Brucker et al. (2008b) found on one wild caught *P. cinereus* a I3C concentration of $51\mu M$, but a larger sample size is needed to confirm I3C concentration on amphibian skin. Violacein is a dark violet pigment that was first identified to be produced by *Chromobacterium violaceum* (Liu and Nizet 2009), which is a close relative to *J. lividum*. It has been shown to have strong antioxidant properties and has the capacity to induce apoptosis (Liu and Nizet 2009). Violacein has been identified with antifungal (Becker et al. 2009, Brucker et al. 2008b), antibacterial, antiprotozoan (Liu and Nizet 2009), antiviral, and antitumorigenesis (Antonisamy and Ignacimuthu 2010). Brucker et al. (2008b) identified that violacein was inhibitory against *B. dendrobatidis* using a 96-well inhibition assay ($MIC=1.8\mu M$). They also identified that there appears to be a threshold concentration of violacein needed to

inhibit *B. dendrobatidis* *in vitro*. Becker et al. (2009) determined that the concentration of violacein on the skins of *P. cinereus* that was protective against *B. dendrobatidis* was approximately 18 μ M and Brucker et al. (2008b) found concentrations of violacein on three naturally caught *P. cinereus* to be 207 μ M, 10 μ M, and 4 μ M respectively.

For this study I used these three metabolites at inhibitory concentration of 50% (IC₅₀) and minimum inhibitory concentration (MIC) with the exception of violacein. Since there was no identified IC₅₀ with violacein, I used the concentration of violacein found to be protective against *B. dendrobatidis* determined on the salamander *P. cinereus*. Natural concentrations of these metabolites found on amphibian skins needs to be researched more with larger sample sizes and with more than one amphibian species.

The Role of Chemotaxis of B. dendrobatidis in its Transmission and Re-Infection

To investigate how chemotaxis can influence transmission and re-infection of *B. dendrobatidis*, I mathematically illustrated zoospore movement using a random walk on a simple lattice with parameters gathered from my three metabolite investigations. With this model I illustrate that if a zoospore is in water near an amphibian is it more likely that the zoospore will move towards the amphibian (initial infection) or away (transmission) depending on the metabolite concentration present on the amphibian. Likewise, I illustrate that a zoospore on the amphibian skin is more likely to move towards the amphibian (re-infection) or away from that amphibian to infect a susceptible host (transmission) dependant on metabolite concentration present on the already infected amphibian.

This model can be important in understanding the lethality-threshold of *B. dendrobatidis* and can provide insights in developing mathematical models that include re-infection parameters as well as varying proportions of populations that have anti-*B. dendrobatidis* bacteria on amphibian skins. If metabolite concentrations on amphibian skins are high they may provide the amphibian with protection from *B. dendrobatidis* by encouraging zoospores to move away and not colonize. This may lead to zoospores moving towards amphibians without anti-*B. dendrobatidis* bacteria. This could quickly cause mortality of susceptible amphibians if a large number of zoospores move towards it and if zoospore density surpasses the lethality-threshold (Carey et al. 2006). Likewise this model may also predict that if metabolite concentrations are present on the amphibian but not high enough to provide complete protection from *B. dendrobatidis* through mortality of the pathogen, then it may be possible to maintain *B. dendrobatidis* infections below mortality threshold resulting in the endemic populations of amphibians.

Methods

Maintaining Batrachochytrium dendrobatidis Cultures

Two isolates of *B. dendrobatidis*, JEL 310 and JEL 423, used for this project were received from Dr. Joyce Longcore at the University of Maine. Isolate JEL 310 was isolated from the frog species *Smilisca phaeota* from Fortuna, Panama by P. Murphy and had been cultured from -80°C frozen stock for approximately a year before the beginning of this experiment. The isolate JEL 310 has been shown to have morbidity and mortality effects on *Plethodon cinereus* (Becker et al. 2009). The isolate JEL 423 was isolated from the frog species *Phyllomedusa lemur* that was collected during a die-off in Panama and had been cultured from -80°C frozen stock approximately one month before the beginning of its use.

Stock cultures were serially transferred on a weekly basis for both JEL 310 and JEL 423. Both isolates of *B. dendrobatidis* were maintained in 1% tryptone broth and stored at 23°C. Each week 1mL of culture was transferred to a fresh stock of tryptone broth.

Additional stock cultures containing antibiotics were serially transferred on a monthly schedule. Both isolates of *B. dendrobatidis* were maintained in 1% tryptone broth + 50µL of penicillin (100mg/mL) and streptomycin sulfate (200mg/mL) each. Cultures were allotted a week of growth at 23°C and then stored at 4°C for approximately a month. Each month 1mL of the culture was transferred to fresh antibiotic broth. These cultures were maintained in the event that stock cultures maintained on a weekly basis in the absence of antibiotics were compromised. In the event new weekly cultures were needed, 1mL of the monthly stock that contained antibiotics was transferred to fresh 1%

tryptone broth and allowed to grow one week before another transfer to 1% tryptone broth. After two serial transfers into fresh 1% tryptone, isolates were then used for experimentation to ensure that there were no effects from being maintained in antibiotics.

Harvesting B. dendrobatidis Zoospores

In order to harvest *B. dendrobatidis* zoospores, 1mL of weekly *B. dendrobatidis* stock was transferred to a 1% agar + 1% tryptone plate and evenly spread on the entire surface of the plate. Plates were allotted time to just dry (approximately 5-10 minutes), covered and sealed, and placed at 23°C for approximately 3-5 days. After 3-5 days of growth, plates were observed under a compound microscope at 200x to confer active zoospores.

Plates that had activity were then flooded with 3mL of sterile Provosoli medium (Wyngaard and Chinnappa, 1982) for 20 minutes to stimulate *B. dendrobatidis* zoosporangia to release zoospores. Another 1mL of Provosoli medium was added to the plates and gently “swished” around the plate to bath the zoosporangium. All standing liquid containing the released zoospores was then gently removed off of the plate using a pipette and stored in 15mL Falcon tubes.

Active zoospores, defined as actively moving, were then counted using a standard hemocytometer slide. To reach target densities of approximately 450-500 active zoospores/100µL Provosoli medium, active zoospore broths were either concentrated or diluted accordingly.

Customized Microscope Slides

Standard glass microscope slides were fashioned with eight 1cm x 1cm chambers using 100% Silicone Aquarium Sealant (All-Glass Aquarium Co., Inc., Franklin, WI) (Figure 6). These chambers were made by hand using a standard grid. The silicone was given at least 12 hours to set while in a sterile, closed container.

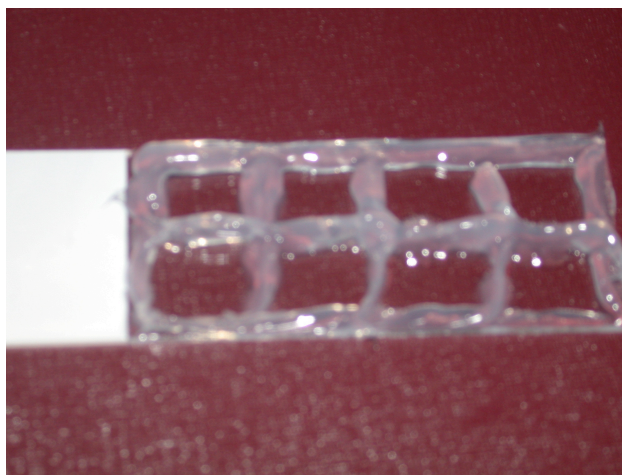


Figure 6. Customized microscope slide with 8 chambers approximately 1cm x 1 cm made out of 100% Silicone Aquarium Sealant.

Immediately before an experiment 40 μ L of substrate was plated along the left edge of each chamber. The experimental treatments were: 1% agar + 1% tryptone (**control**) and 1% agar + 1% tryptone with one of the following: 68.89 μ L indole-3-carboxaldehyde (**I3C**) (minimum inhibitory concentration (MIC)), 136.13 μ L 2,4-diacetylphloroglucinol (2,4,-DAPG), which is the MIC (**2,4-DAPG high**), 8.73 μ L 2,4-DAPG, which is the IC₅₀ (**2,4-DAPG low**), 1.8 μ L violacein, which is the MIC (**violacein low**), and 18 μ L violacein (**violacein high**) (Table 1). For all metabolites with

the exception of violacein the *B. dendrobatidis* isolate JEL 310 was studied. The change in isolate use was due to the mortality of isolate JEL 310 and the exhaustion of available replacements. The *B. dendrobatidis* isolate JEL 423 was used to study chemotaxis in the presence and absence of violacein. Substrates were given a few minutes to polymerize before a solution of 450-500 active zoospores of *B. dendrobatidis* per 100 μ L Provosoli medium was added to a chamber. Vaseline was added around the silicone chambers to ensure there was sealing between all chambers and a 24 x 60 mm plastic microscope cover slip was used to seal top of chambers.

Table 1. A complete table of metabolite impregnated into 1% agar + 1% tryptone, the concentration used (μL), and the *B. dendrobatidis* isolate used.

Impregnated Metabolite (in 1% agar + 1% tryptone)	Concentration (μL)	Isolate
No Metabolite (Control)	-	JEL 310
I3C (MIC)	68.89	JEL 310
2,4-DAPG (MIC)	136.13	JEL 310
2,4-DAPG (IC_{50})	8.73	JEL 310
No Metabolite (Control)	-	JEL 423
Violacein (MIC)	1.8	JEL 423
Violacein (IC_{50})	18	JEL 423

Note. The isolate JEL 423 was used to study chemotaxis in the presence and absence of violacein. The change in isolate was due to the loss of isolate JEL 310.

Data Collection of Zoospore Movement

Chambers were observed at two times: initial time ($t = 0$) and 6 hours at 200x using a Nikon eclipse TE2000 inverted microscope. Sampling time was determined by calculating the proportion of *B. dendrobatidis* zoospores that were still active every hour for 12 hours. This was done by randomly sampling ten 100 μ m x 100 μ m grids using a random number generator and calculating the average proportion of active zoospores for each hour. Sampling time was determined to be initial time ($t = 0$) and the time where approximately 50% of *B. dendrobatidis* isolate 310 zoospores remained active. The substrate was sometimes placed to appear on the left and other times to appear on the right to account for any effects due to light. Data were collected by taking 50 still-frame photos over a 10 second period (approximately every 0.2 seconds) taken with a Roper Scientific CoolSnap HQ monochrome digital camera focused at the center width of the chambers at approximately the center depth along the edge of the substrate and of interest area (Figure 7). These images were collected using Roper Scientific Image Capture Software (Roper Scientific, Germany).

Zoospore movement was collected by haphazardly selecting 5 zoospores from each of the three distances away from the edge of the substrate at both $t = 0$ hours and $t = 6$ hours. The distances used were 0-100 μ m, 200-300 μ m, and 500-600 μ m. To put this in context, the far end of the silicone was 5000 μ m. Each selected zoospore was followed throughout each still-frame for 10 seconds. At each transition between still-frames, which was approximately every 0.2 seconds, the movement of the zoospore either towards the substrate, no movement, or away from the substrate was recorded.

Initial distance of the zoospore to the edge of the agar source (agar/Provosoli interface) was also recorded. After collecting movement data, end point distance of the zoospore to the edge of the agar source was also recorded. Total distance moved by a zoospore over 10 seconds was calculated.

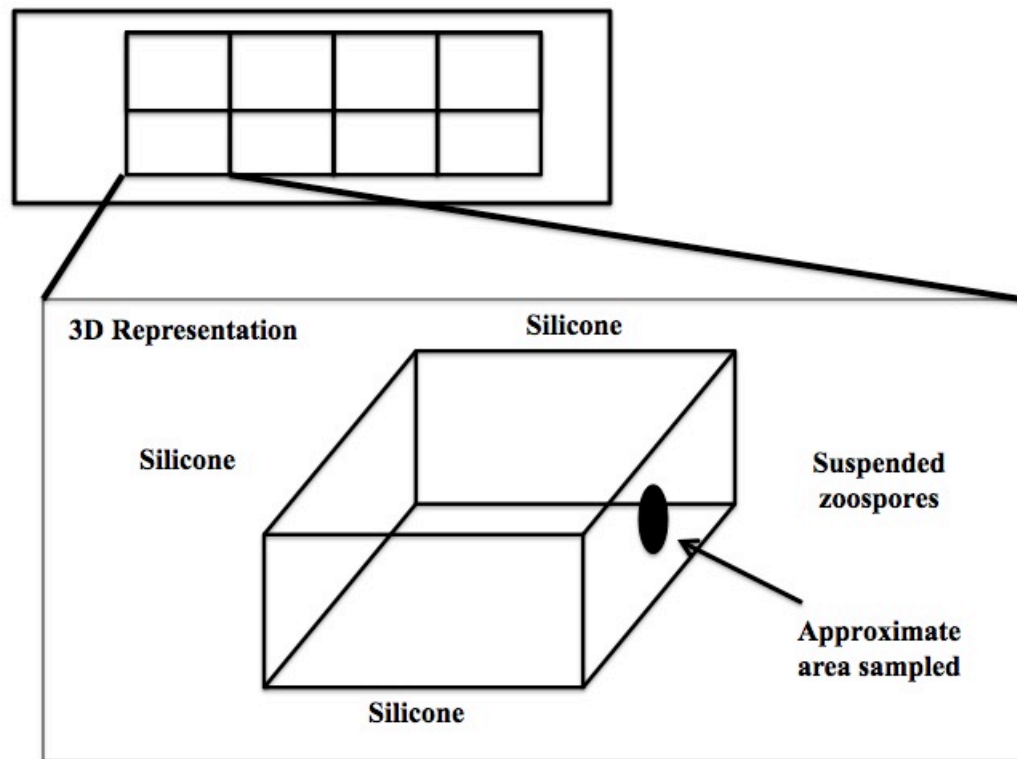


Figure 7. A schematic of the 3D area within a chamber that was used for sampling *B. dendrobatidis* zoospore movement. The camera was focused in the center of the substrate at central depth along the edge of the substrate and area where suspended zoospores were plated. The 3D box in the figure represents the substrate.

The number of active zoospores was counted at both 0-100 μ m and 500-600 μ m distances away from the substrate and compared between $t = 0$ hours and $t = 6$ hours.

Statistics

Zoospore movement data were statistically analyzed to determine if movement towards substrate and away from substrate was a function of time, location, and treatment using ANOVA. The number of movements made towards and away from the substrate was analyzed only. The no movement variable was not analyzed since it was determined by the other two variables. Each data point used for statistical analysis was an average of all zoospores recorded within a time, location and treatment combination. The averaging procedure eliminated the possibility of “pseudo-replication” because the responses of potentially interacting zoospores within a chamber were averaged. This left the level of replication as five, which was the number of chambers used for each treatment. In addition, according to the central limit theorem those means should approach a normal distribution. If time and location were found to not have a significant effect on movement towards substrate and away from substrate, they were pooled together and tested using a one-way ANOVA to determine the effect of treatment.

The number of active zoospores at two locations (0-100um and 500-600um) and at two time points ($t = 0$ and 6 hours) were compared using ANOVA. If location had no effect on the number of active zoospores then these data were pooled.

Mathematical Model

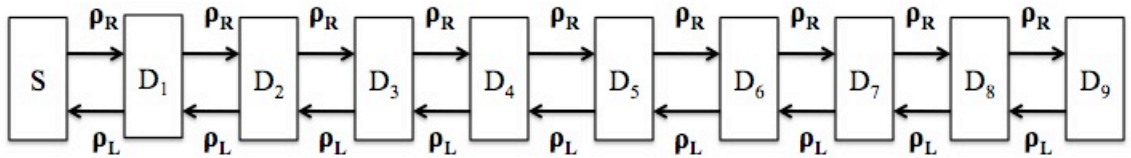


Figure 8. Schematic showing a model structure of *B. dendrobatidis* movement away from or towards a source (S) at increasing starting distances from the source. All transitional probabilities are fully defined in Table 1.

A random walk on a simple lattice was modeled with a system of difference equations describing *B. dendrobatidis* zoospore movement away from or towards a source (S) depending on its starting distance from the source. The source represents the 1% agar + 1% tryptone (control) or 1% agar + 1% tryptone + metabolite (experimental). The following nine boxes represent an increasing distance away from the source (S). Since we will later find location was found to have no effect on zoospore movement, the probability of a zoospore moves to the left (ρ_L) or to the right (ρ_R) during the time increment of 10 seconds are the same no matter what location the zoospore is in. A schematic of the model is shown in Figure 8 and a full set of equations is given as follows:

$$S(t+1) = \rho_L D_1 - \rho_R S \quad (1.1)$$

$$D_1(t+1) = \rho_L D_2 + \rho_R S - \rho_L D_1 - \rho_R D_1 \quad (1.2)$$

$$D_2(t+1) = \rho_L D_3 + \rho_R D_1 - \rho_L D_2 - \rho_R D_2 \quad (1.3)$$

$$D_3(t+1) = \rho_L D_4 + \rho_R D_2 - \rho_L D_3 - \rho_R D_3 \quad (1.4)$$

$$D_4(t+1) = \rho_L D_5 + \rho_R D_3 - \rho_L D_4 - \rho_R D_4 \quad (1.5)$$

$$D_5(t+1) = \rho_L D_6 + \rho_R D_4 - \rho_L D_5 - \rho_R D_5 \quad (1.6)$$

$$D_6(t+1) = \rho_L D_7 + \rho_R D_5 - \rho_L D_6 - \rho_R D_6 \quad (1.7)$$

$$D_7(t+1) = \rho_L D_8 + \rho_R D_6 - \rho_L D_7 - \rho_R D_7 \quad (1.8)$$

$$D_8(t+1) = \rho_L D_9 + \rho_R D_7 - \rho_L D_8 - \rho_R D_8 \quad (1.9)$$

$$D_9(t+1) = \rho_R D_8 - \rho_L D_9 \quad (1.10)$$

The model was implemented stochastically to illustrate how many steps a single zoospore would take to reach the source. A direct simulation was created to simulate zoospores moving on a lattice in order to measure the number of steps a zoospore took to reach the source. This model was simulated 500 times once for each treatment with one zoospore at starting position farthest away from the source using a probability matrix. The mathematical equations (1.1-1.10) describe the probability for a single zoospore to be at a certain location after t time steps. Simulations then describe a zoospores random movement along the lattice using the described probabilities given by equations 1.1-1.10. The number of movements it took for a zoospore to reach the source was recorded and the weighted average for each treatment was found.

The model was also implemented deterministically to illustrate the dispersal of zoospores if their starting position is at the middle of the model (D_5). The overall distribution was then tracked as a function of time and recorded. This model was performed for each treatment once with 200 zoospores beginning at D_5 . The model was stopped at six different time steps: initial time step where all 200 zoospores start at D_5 and every 10 time steps until 50 times steps are reached. The number of zoospores at each position was recorded and the weighted average for each treatment was found.

The mathematical software Sage® (www.sagemath.org) was used to implement all models. Zoospores with a starting position farthest away from the source would correspond to zoospores at a mucus/pond interface and would simulate zoospores that would be transmitted from either pond water or from an infected amphibian. Zoospores starting on the source (S) would be similar to zoospores released to the surface of the epidermis that could re-infect.

Parameter Estimation

Parameters are fully defined with estimated values from my experiment in Table 2. Each parameter was estimated by finding the probability of a zoospore moving towards or away from the source. Since location was found to not have an effect on zoospore movement, the probabilities for a zoospore moving from each box either towards or away from the source is the equal for all distances.

Table 2. Model parameter descriptions, symbols, and values.

Parameter Description	Symbol	Control (310); (423)	DAPG (L); (H)	IC (H)	Violacein (L); (H)
Probability that a zoospore to the left into the next location	ρ_L	0.408; 0.404	0.350; 0.380	0.371	0.383; 0.385
Probability that a zoospore to the right into the next location	ρ_R	0.305; 0.428	0.491; 0.498	0.409	0.581; 0.429

Results

Description of Batrachochytrium dendrobatidis Zoospore Movement Patterns

Both isolates of *B. dendrobatidis*, JEL 310 and JEL 423, displayed several patterns of zoospore movement (Figure 9A-D). These movement patterns were observed in all treatments including the control, and there were no distinguishing movement patterns that were typical to an experimental treatment. Zoospores typically were seen to move in circular patterns (Figure 9A) or were seen oscillating inwards or outwards (Figure 9B). Less often, zoospores were seen to display linear movement either with direction (Figure 9C) or without direction (Figure 9D) in a seemingly random nature. Linear non-directional movement was observed to be the most rare pattern of zoospore movement.

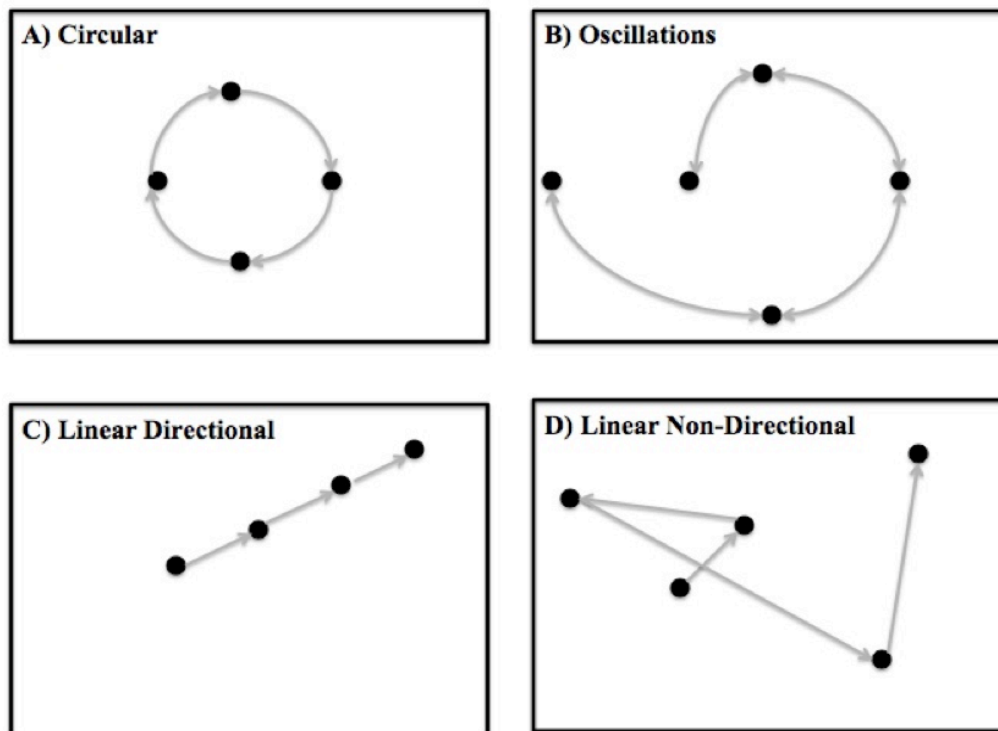


Figure 9. These are movement patterns observed by *B. dendrobatidis* zoospores (JEL 310 and 423) in all treatments including the control. Patterns most frequently observed were circular (A) and inward/outward oscillations (B). Less commonly, zoospores were also seen to move linearly with direction (C) or without direction (D).

Determining Sampling Times

Sampling times were determined to be initial time ($t = 0$) and the time at which 50% of *B. dendrobatidis* zoospores remained active for isolate JEL 310 (Figure 10A) ($n = 3$). This isolate chosen was initially to be used for the entire experiment. Approximately 50% of zoospores remained active up to 6 hours after harvest and by the twelfth hour the vast majority of zoospores were no longer active. When observed at 24 hours, zoosporangium could be seen suggesting that inactive zoospores were not dead but

transition life stage. Activity was also measured for isolate JEL 423 (Figure 10B) where by the twelfth hour approximately 75% of zoospores remained active ($n = 3$).

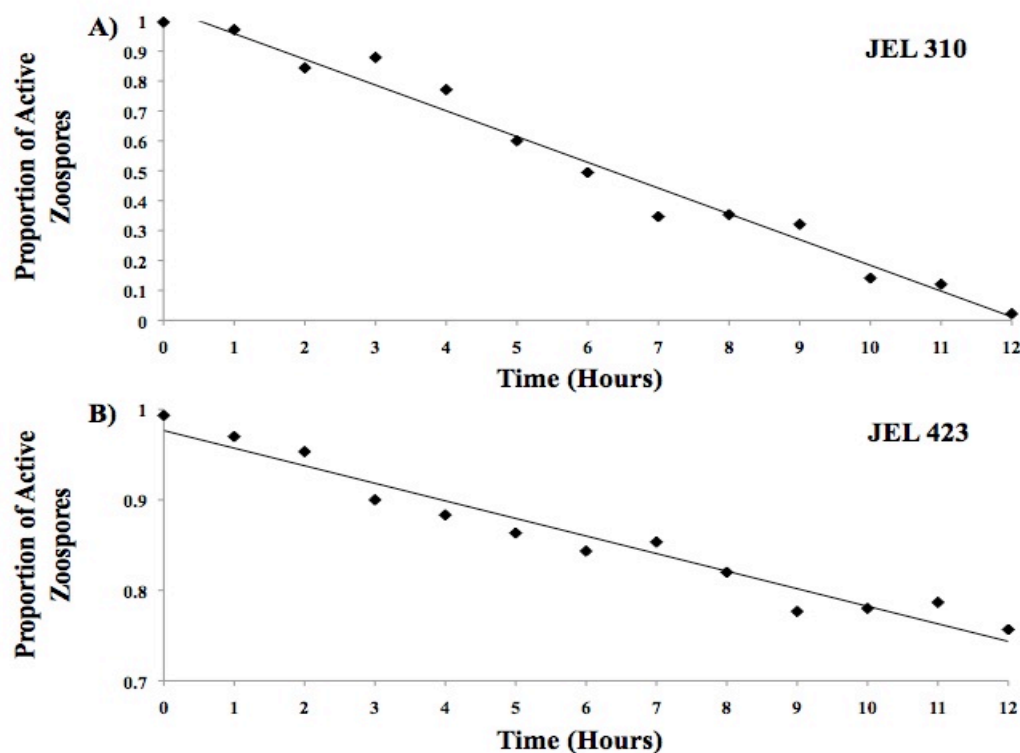


Figure 10. Zoospore activity for *B. dendrobatidis* isolates JEL 310 (A) ($n = 3$) and JEL 423 (B) ($n = 3$). Sampling times were determined to be initial ($t = 0$) and where 50% of JEL 310 zoospores remained active ($\sim t = 6$).

Synopsis of Results

Zoospores from isolate JEL 310 moved toward the source less in both 2,4-DAPG low and 2,4-DAPG high treatments than the control treatment, but not the I3C treatment. For all treatments (2,4-DAPG low, 2,4-DAPG high, and I3C), zoospores from isolate JEL 310 moved away from the metabolite more than the control treatment. The mathematical

models predicted that zoospores would reach the source in the control treatment faster than zoospores in 2,4-DAPG low, 2,4-DAPG high, and I3C treatments and that more zoospores would distribute onto the source in the control than all three treatments.

There was no difference in the number of movements zoospores from isolate JEL 423 made either toward or away from both violacein low and violacein high compared to the control treatment. The mathematical models predicted that zoospores in the control, violacein low, and violacein high would all reach the source at approximately the same time and that zoospores would distribute equally in the violacein high and control treatment. Less zoospores would distribute on the source in the violacein low treatment compared to violacein high and control treatments.

B. dendrobatidis (JEL 310) Zoospore Movement Toward and Away from the Source

Within treatment comparisons

There was no overall significant effect of time ($t = 0$ hours versus $t = 6$ hours) on zoospore movement patterns and total distance a zoospore moved towards or away from the source during a 10 second time period within any of the treatments (Table 3). Time had no effect on zoospore movement towards the source within the control, 2,4-DAPG low, 2,4-DAPG high, and I3C treatments (Table 3). Time had no effect on zoospore movement away from the source within the control, 2,4-DAPG low, 2,4-DAPG high, and I3C treatments (Table 3). Time also had no effect on total distance a zoospore moved during a ten second period of time within the control, 2,4-DAPG low, 2,4-DAPG high, and I3C treatments (Table 3). Since there was no effect of time on zoospore movement

either towards or away from the source or on total distance a zoospore moved these data were pooled together.

Table 3. The effect of time on *B. dendrobatidis* (JEL 310) movement within treatment comparisons towards and away from the source and total distance a zoospore moved within a time period.

ANOVA				
<i>Movement towards the source</i>				
<i>Treatment</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control	10	28	0.24	0.0639
2,4-DAPG low	10	28	0.02	0.8994
2,4-DAPG high	10	28	0.82	0.3731
I3C	6	18	0.10	0.7206
<i>Movement away from the source</i>				
<i>Treatment</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control	10	28	3.77	0.1262
2,4-DAPG low	10	28	0.47	0.4943
2,4-DAPG high	10	28	0.03	0.8573
I3C	6	18	0.10	0.7590
<i>Total distance a zoospore moved during a 10 sec. period</i>				
<i>Treatment</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control	10	28	0.24	0.6302
2,4-DAPG low	10	28	3.08	0.7223
2,4-DAPG high	10	28	0.53	0.4733
I3C	6	18	1.79	0.9516

Location (0-100µm, 200-300µm, and 500-600µm) had no effect on zoospore movement towards the source within the control, 2,4-DAPG low, and I3C treatments (Table 4). Zoospores moved more towards the metabolite as location from the source increased within the 2,4-DAPG high treatment ($P = 0.0025$) (Table 4) (Figure 11A).

Location had no effect on zoospore movement away from the source within the control, 2,4-DAPG low, and I3C treatments (Table 4). Zoospores moved away from the metabolite less frequently as location from the source increased within the 2,4-DAPG high treatment ($P = 0.0200$) (Table 4) (Figure 11 B).

Sampling location did not have a significant effect on zoospore total movement over a ten second period of time within the control, 2,4-DAPG low, and I3C treatments (Table 4). There was an effect of location on zoospore total movement within the 2,4-DAPG high treatment, where zoospores moved further away from the metabolite at sampling locations 0-100µm and 200-300µm than zoospores sampled at 500-600µm ($P = 0.0001$). Locations were pooled for all treatments, including 2,4-DAPG high, since the overall trend was for the response variables to not vary as a function of location.

Table 4. The effect of location on *B. dendrobatidis* (JEL 310) movement within treatment comparisons towards and away from the source and total distance a zoospore moved within a time period.

ANOVA				
<i>Movement towards the source</i>				
<i>Treatment</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control	10	27	0.05	0.9540
2,4-DAPG low	10	27	2.57	0.9720
2,4-DAPG high	10	27	7.79	0.0025
I3C	6	15	1.46	0.2707
<i>Movement away from the source</i>				
<i>Treatment</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control	10	27	0.02	0.9777
2,4-DAPG low	10	27	0.86	0.4346
2,4-DAPG high	10	27	4.62	0.0200
I3C	6	15	0.50	0.6202
<i>Total distance a zoospore moved during a 10 sec. period</i>				
<i>Treatment</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control	10	27	0.50	0.6150
2,4-DAPG low	10	27	3.08	0.0645
2,4-DAPG high	10	27	7.79	0.0001
I3C	6	15	1.79	0.2088

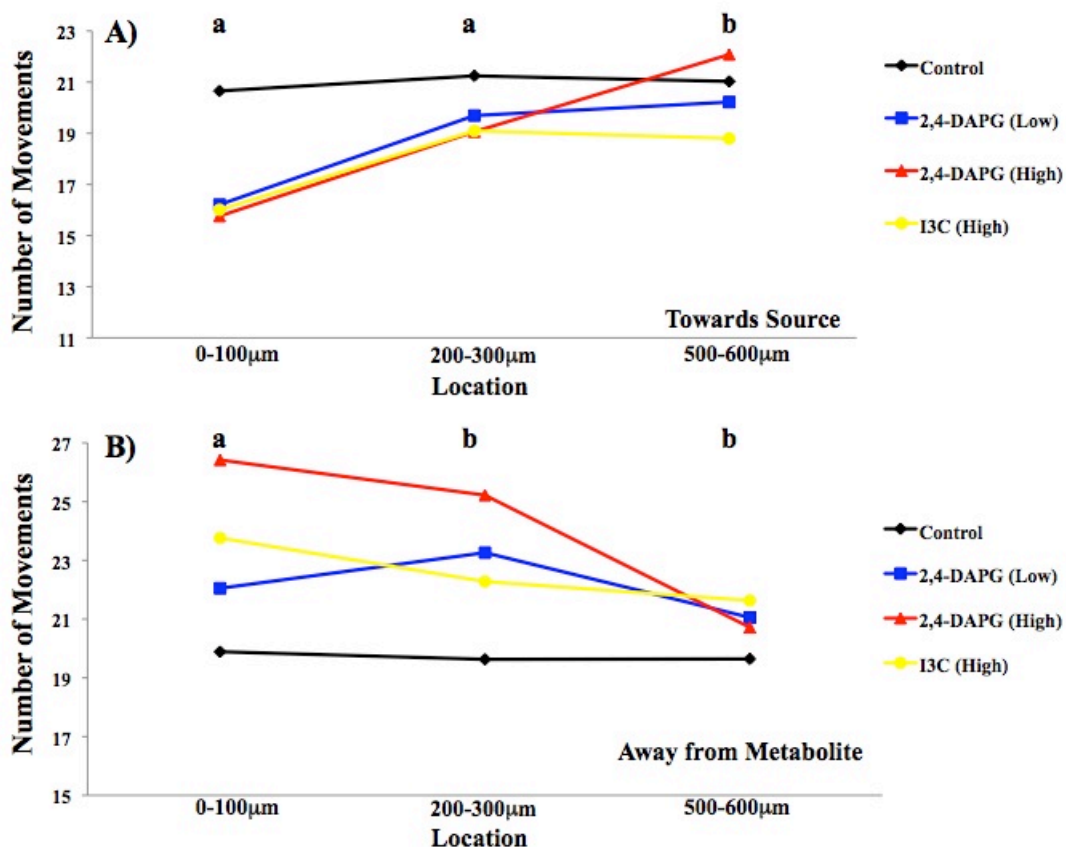


Figure 11. Average *B. dendrobatidis* (JEL 310) zoospore movement over two time periods (0 and 6 hours) toward the source (A) and away from the source (B) for control (n's = 10), I3C (n's = 6), 2,4-DAPG low (n's = 10), and 2,4-DAPG high (n's = 10) respectively at each sampling location. Location only had a significant effect on 2,4-DAPG high both towards the metabolite ($P = 0.0025$) and away from the metabolite ($P = 0.0200$). Letters above the graphs represent Tukey groupings for 2,4-DAPG high.

Between treatment comparisons

Movement towards the source

Treatment had a significant effect on zoospore movement towards the source.

Zoospores in the control moved towards the source significantly more than 2,4-DAPG at low and high treatments ($P < 0.0001$) (Table 5) (Figure 12A). Both 2,4-DAPG low and high treatments were grouped together and apart from the control treatment as determined

by Tukey groupings. There was no significant effect on zoospore movement towards the source between the control and I3C treatments although there was a trend for lower number of movements towards the I3C treatment (Table 5).

Movement away from the source

The treatments 2,4-DAPG low, 2,4-DAPG high, and I3C had a higher number of moves away from the source containing the metabolite relative to the control that contained no metabolite (Figure 12B). Zoospores moved away from the source that contained both 2,4-DAPG at low and high treatments more frequently than the control group ($P = 0.0086$) (Table 5). Zoospores also moved away from the source that contained I3C more frequently than the control group ($P = 0.0149$) (Table 5).

Table 5. Between treatment comparisons of *B. dendrobatidis* (JEL 310) movement towards and away from the source.

ANOVA				
<i>Movement towards the source</i>				
<i>Treatments</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control, 2,4-DAPG low, & 2,4-DAPG high	30	87	11.16	<0.0001
Control & I3C	30, 18	46	3.18	0.0812
<i>Movement away from the source</i>				
<i>Treatments</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control, 2,4-DAPG low, and 2,4-DAPG high	30	87	5.04	0.0086
Control and I3C	30, 18	46	6.40	0.0149

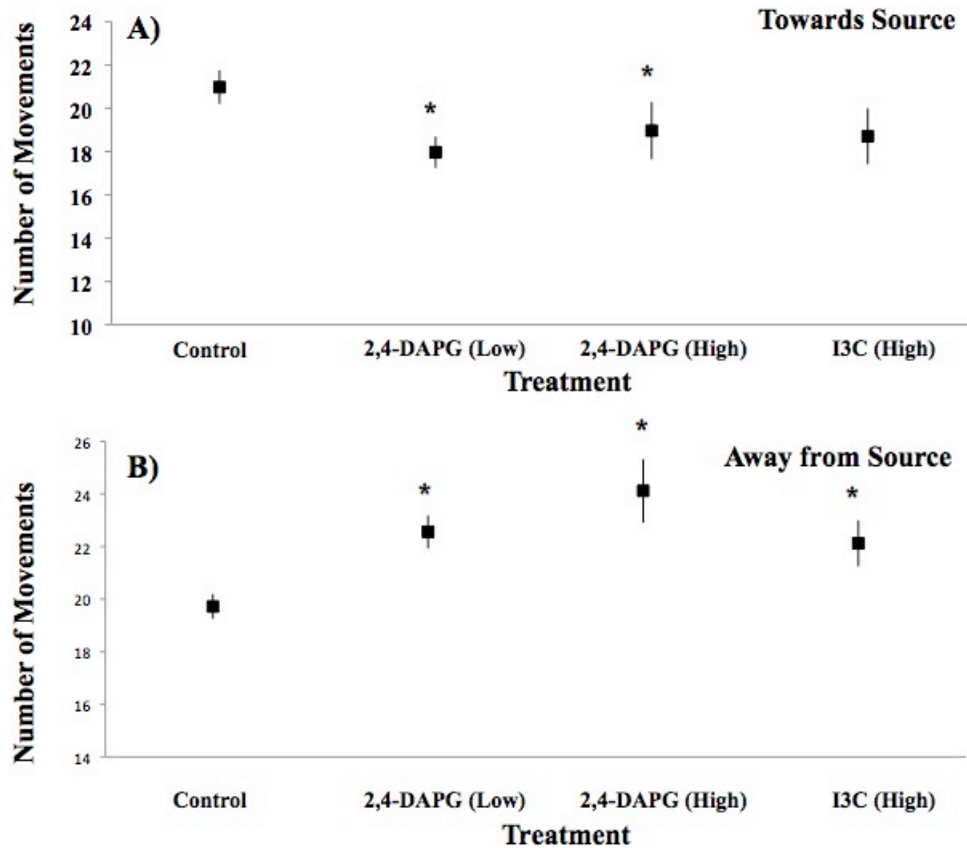


Figure 12. Average *B. dendrobatidis* (JEL 310) zoospore movement over sampling location and time toward the source (A) and away from the source (B) for control (n = 30), 2,4-DAPG low (n = 30), 2,4-DAPG high (n = 30), and I3C (n = 18) respectively. Both 2,4-DAPG low and high treatments moved towards the source less than the control ($P < 0.0001$) and away from the source more relative to the control ($P = 0.0086$). Zoospores in the I3C treatment did not move away from the source more than the control ($P = 0.0812$), but did move away from the source more relative to the control ($P = 0.0149$). Asterisk above points are significantly different from the control and error bars represent standard error.

B. dendrobatidis (JEL 423) Zoospore Movement Toward and Away from the Source

B. dendrobatidis (JEL 423) zoospore movement data were only collected for initial time ($t = 0$ hours). It was determined from the results using isolate 310 that time had no effect on zoospore movement either towards or away from the source or on total zoospore movement for a ten second period of time relative to $t = 6$ hours. Therefore, $t = 6$ hours was not sampled.

Within treatment comparisons

Location (0-100 μ m, 200-300 μ m, and 500-600 μ m) had no effect on zoospore movement towards the source within the control, violacein low, and violacein high treatments (Table 6). Location also had no effect on zoospore movement away from the source within the control, violacein low, and violacein high (Table 6). During a period of ten seconds, location had no effect on total zoospore movement towards or away from the source within the control, violacein low, and violacein high (Table 6). These data were then pooled together.

Table 6. The effect of location on *B. dendrobatidis* (JEL 423) movement within treatment comparisons towards and away from the source and total distance a zoospore moved within a time period.

ANOVA				
<i>Movement towards the source</i>				
<i>Treatment</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control	5	12	0.50	0.6196
Violacein low	5	12	1.52	0.2575
Violacein high	5	12	0.25	0.7810
<i>Movement away from the source</i>				
<i>Treatment</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control	5	12	0.30	0.7475
Violacein low	5	12	0.01	0.9867
Violacein high	5	12	2.67	0.1100
<i>Total distance a zoospore moved during a 10 sec. period</i>				
<i>Treatment</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control	5	12	1.32	0.3041
Violacein low	5	12	0.72	0.5075
Violacein high	5	12	2.17	0.1565

Between treatment comparisons

The control and both violacein low and high concentration had approximately equal movements both towards and away from the source (Table 7) (Figure 13A-B).

Treatment also had no effect on total distance a zoospore moved towards or away from the source among the control and both violacein low and high treatments (Table 7).

Table 7. Between treatment comparisons of *B. dendrobatidis* (JEL 423) movement towards and away from the source and total movement of a zoospore during a time period.

ANOVA				
<i>Movement towards the source</i>				
<i>Treatments</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control, violacein low, & violacein high	15	42	0.44	0.6470
<i>Movement away from the source</i>				
<i>Treatments</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control, violacein low, & violacein high	15	42	0.71	0.5005
<i>Total distance a zoospore moved during a 10 sec. period</i>				
<i>Treatments</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control, violacein low, & violacein high	15	42	0.07	0.9287

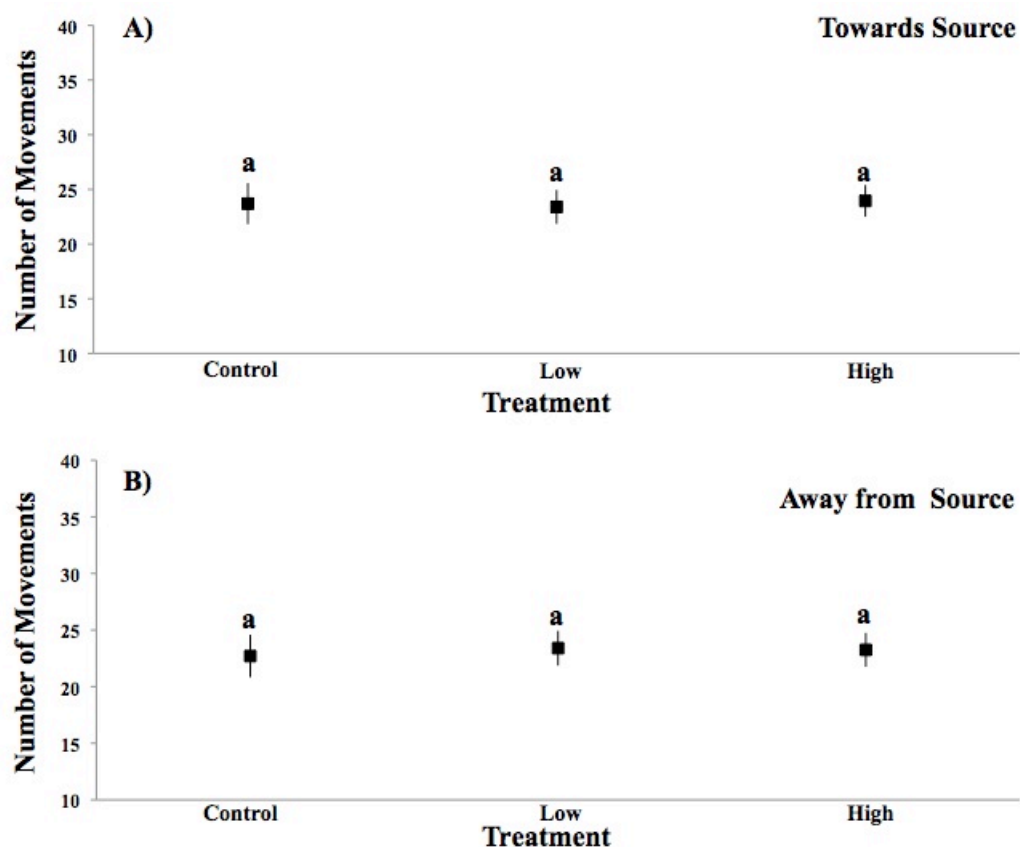


Figure 13. Treatment had no effect on the number of movements a *B. dendrobatidis* (JEL 423) zoospore took either towards (A) the source or away (B) from the source among the control and both violacein low and high treatments. Letters represent Tukey groupings and error bars represent standard error.

***B. dendrobatidis* (JEL 310) Zoospore Activity**

Within treatment comparisons

More zoospores were active at $t = 0$ hours than $t = 6$ hours within the control ($P < 0.0001$), 2,4-DAPG low ($P < 0.0001$), 2,4-DAPG high ($P = 0.0003$), and I3C ($P = 0.0002$) treatments (Table 8) (Figure 14A-B and Figure 15A-B). Location (0-100 μ m and 500-600 μ m) had no effect on the number of active zoospores within the control, 2,4-

DAPG at low, and I3C treatments (Table 8). There were more active zoospores at 500-600µm than at 0-100µm within the 2,4-DAPG high treatment ($P = 0.0005$) (Table 8) (Figure 15B). Locations were pooled for all treatments, including 2,4-DAPG high, since the overall trend was for the response variables to not vary as a function of location.

Table 8. Effect of time and location on *B. dendrobatidis* (JEL 310) zoospore activity within treatments.

ANOVA				
<i>Effect of time (t = 0 and t = 6 hours)</i>				
<i>Treatment</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control	10	18	64.21	<0.0001
2,4-DAPG low	10	18	94.85	<0.0001
2,4-DAPG high	10	18	21.52	0.0003
I3C	6	10	40.41	0.0002
<i>Effect of location</i>				
<i>Treatment</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control	10	18	0.18	0.6788
2,4-DAPG low	10	18	1.75	0.2050
2,4-DAPG high	10	18	21.52	0.0005
I3C	6	10	0.05	0.8273

Between treatment comparisons

The control and 2,4-DAPG low treatments had approximately equal number of active zoospores relative to 2,4-DAPG high treatment that had lower numbers of active zoospores ($P = 0.0005$) (Table 9) (Figure 14A-B and Figure 15A). Treatment had no

effect on the number of active zoospores between the control and I3C treatments (Table 9) (Figure 14A and Figure 15B).

Table 9. Between treatment comparisons of *B. dendrobatidis* (JEL 310) zoospore activity.

ANOVA				
<i>Treatments</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control, 2,4-DAPG low, & 2,4-DAPG high	20	57	8.89	0.0005
Control & I3C	20, 12	30	0.02	0.8766

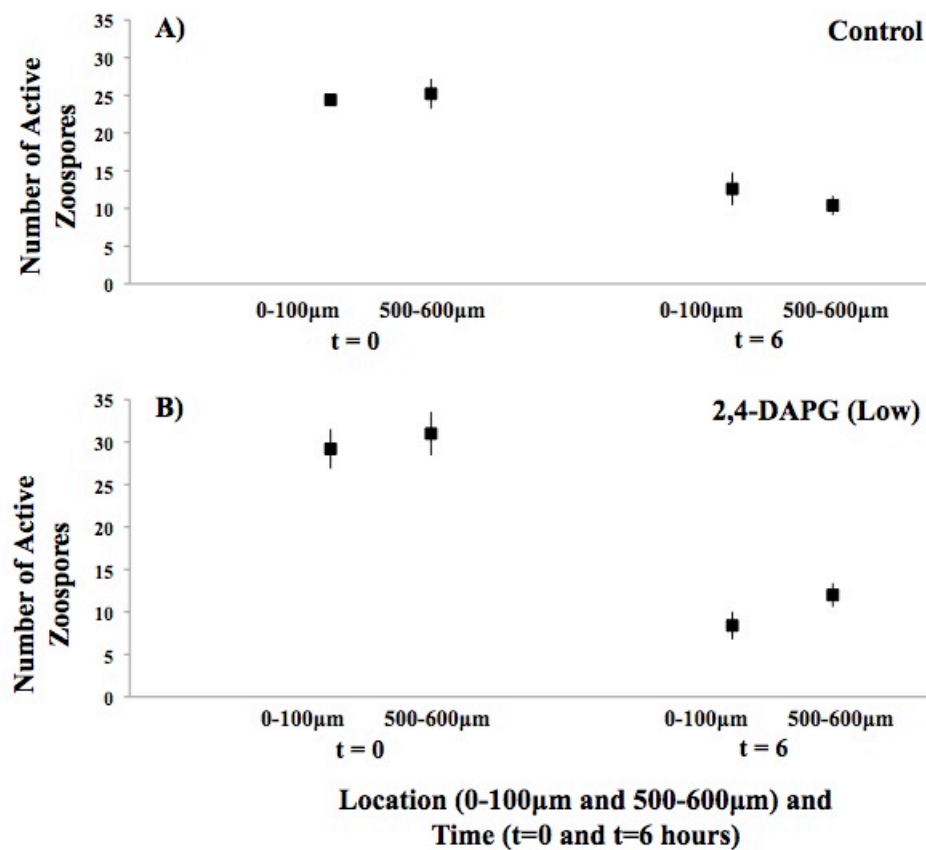


Figure 14. Time had an effect on active zoospores within both the control (n's = 10) and 2,4-DAPG low (n's = 10) ($P < 0.0001$ and $P < 0.0001$ respectively) treatments. The number of active zoospores was approximately equal for both the control and 2,4-DAPG low at both time and location.

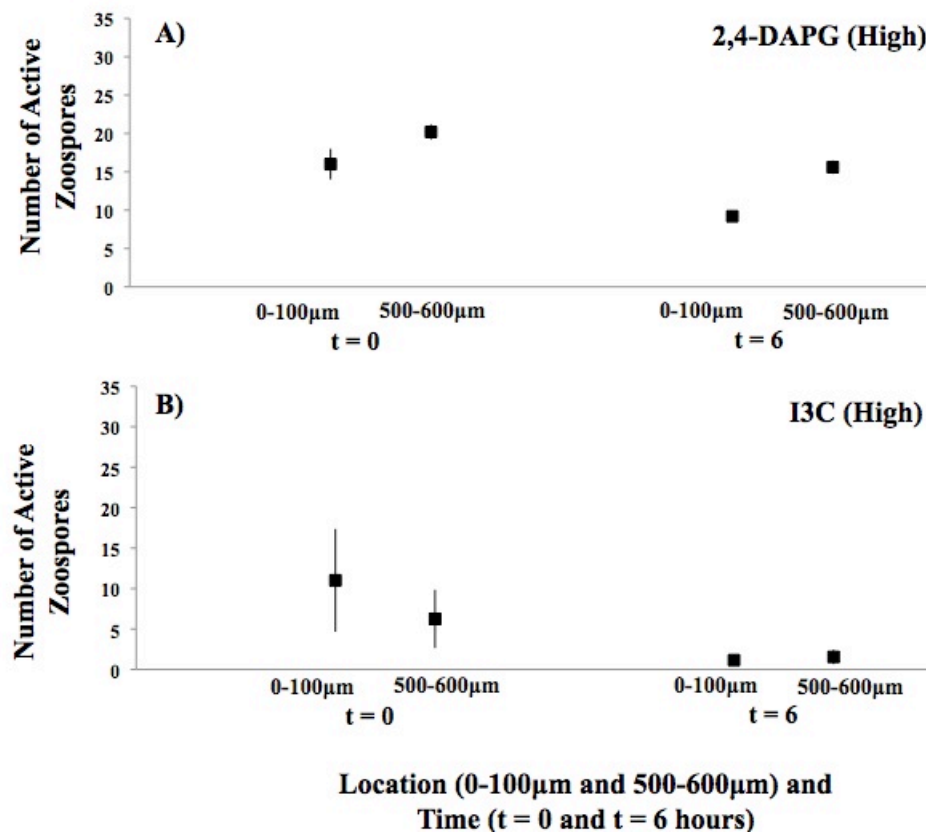


Figure 15. Time had an effect on active zoospores within both the 2,4-DAPG high ($n's = 10$) and I3C ($n's = 6$) ($P = 0.0003$ and $P = 0.0002$ respectively). The number of active zoospores was less 2,4-DAPG high at both time and location relative to both the control and 2,4-DAPG low ($n's = 20$) ($P = 0.0005$).

B. dendrobatidis (JEL 423) Zoospore Activity

Within treatment comparisons

Zoospore activity did not change over two times sampled ($t = 0$ and $t = 6$ hours) within the control, violacein low, and violacein high treatments (Table 10). Location had

no effect on zoospore activity within the control, violacein low, and violacein high treatments (Table 10).

Table 10. Effect of time and location on *B. dendrobatidis* (JEL 423) zoospore activity within treatments.

ANOVA				
<i>Effect of time (t = 0 and t = 6 hours)</i>				
<i>Treatment</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control	5	9	0.09	0.7618
Violacein low	5	9	2.12	0.0782
Violacein high	5	9	0.06	0.6244
<i>Effect of location</i>				
<i>Treatment</i>	<i>n's</i>	<i>df</i>	<i>F</i>	<i>Sig.</i>
Control	5	9	0.09	0.7618
Violacein low	5	9	2.12	0.1632
Violacein high	5	9	0.06	0.6244

Between treatment comparisons

B. dendrobatidis (JEL 423) zoospore activity did not differ between the control (n = 5) and both violacein at low (n = 5) and high (n = 5) concentrations (F = 0.56; DF = 2,12; P = 0.5760).

Mathematical Models (JEL 310)

The number of movements a zoospore took from the furthest starting position (D_9) for simulations that took between 9-100 movements to reach the source can be seen in Figure 16. Not all simulations reached the source between 9-100 movements and took move movements to reach the source. The median number of movements to reach the source was found for simulations in the control to be 12.1. The median number of movements in the 2,4-DAPG low and 2,4-DAPG high treatments was 39.8 and 42.2 respectively. The median number of movements to reach the source in the I3C treatment was 32.2.

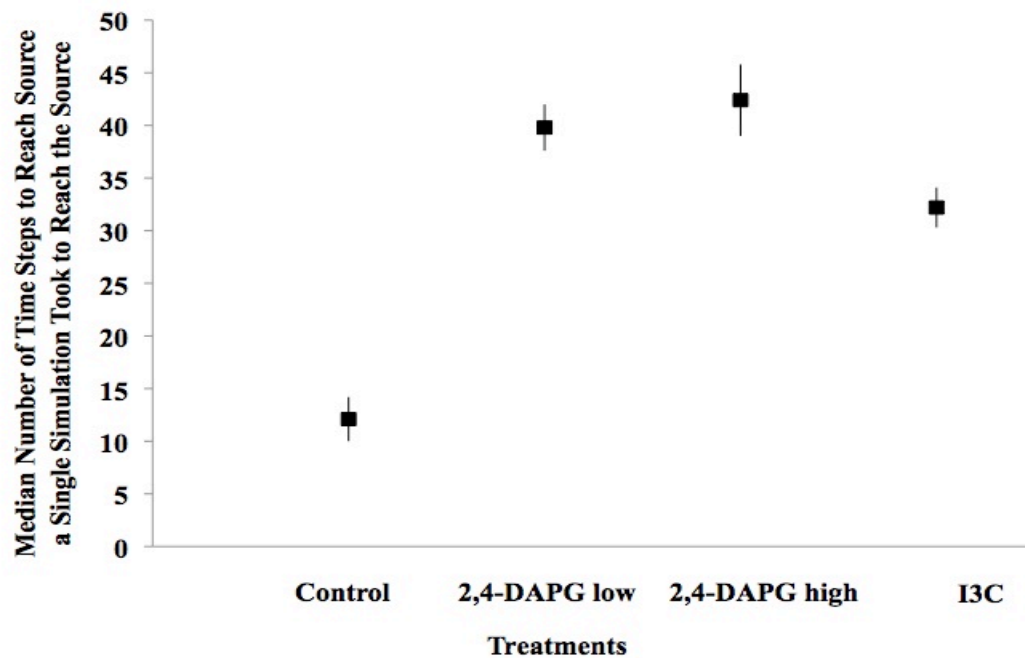


Figure 16. The median number of movements a single *B. dendrobatidis* (JEL 310) zoospore took to reach the source for 500 simulations if released at the location furthest from the source (D_9). Only simulations that reached the source between 9-100 movements are shown.

The model to illustrate zoospore dispersal where 200 zoospores are released at D_5 results are shown in Figure 17. The weighted average is lower in the control compared to the other three treatments (2,4-DAPG low, 2,4-DAPG high, and I3C). Zoospores in the three experimental treatments continue to move away from the source as depicted by the weighted averages increasing as time steps increase. Zoospores in the control treatments continue to move closer to the source as depicted by the weighted averages decreasing as time steps increase.

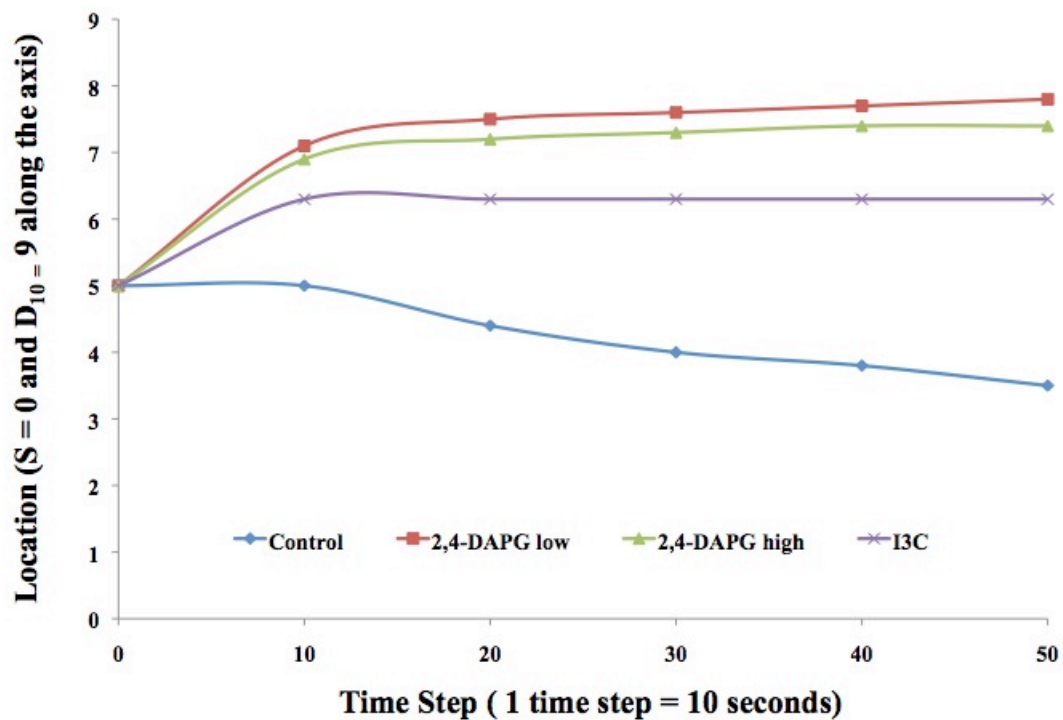


Figure 17. Average number of *B. dendrobatidis* (JEL 310) zoospores at locations in the model at six different time steps (0, 10, 20, 30, 40, 50).

Mathematical Models (JEL 423)

The number of movements a zoospore took from the furthest starting position (D_9) for simulations that took between 9-100 movements to reach the source can be seen in Figure 18. Not all simulations reached the source between 9-100 movements and took move movements to reach the source. The median number of movements simulations from the control treatment took was 23.4 movements. The median number of movements from simulations from the violacein low treatment was 54.3. The median number of movements from the violacein high treatment was 32.2.

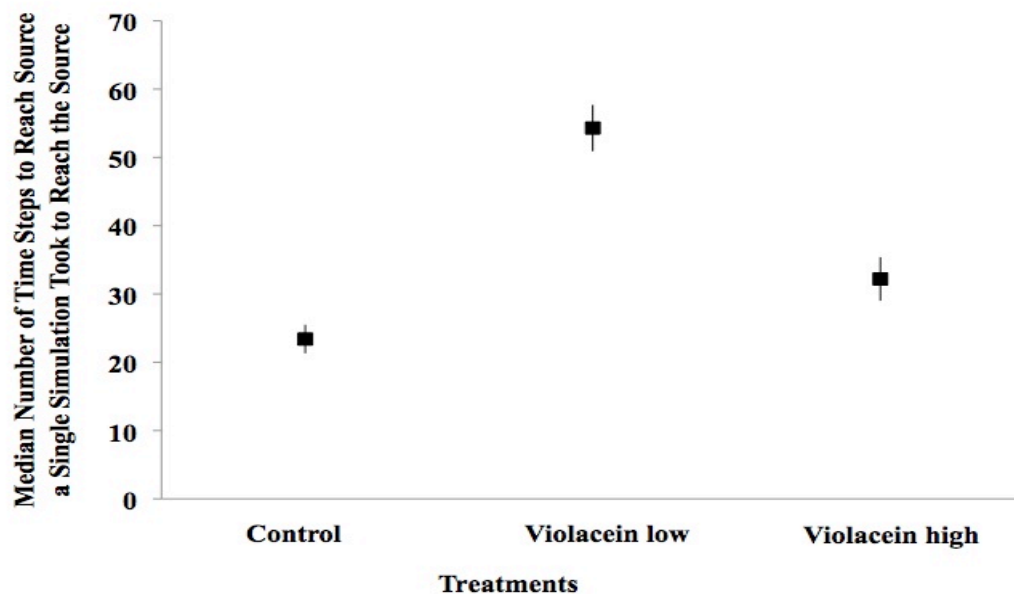


Figure 18. The median number of movements a single *B. dendrobatidis* (JEL 423) zoospore took to reach the source for 500 simulations if released at the location furthest from the source (D_9). Only simulations that reached the source between 9-100 movements are shown.

The model to illustrate zoospore dispersal where 200 zoospores are released at D_5 results are shown in Figure 19. The weighted average is lower in the control and violacein high treatments compared to the violacein low treatment. Zoospores in the control and violacein high treatments continue to move towards the source as time steps increase. Zoospores in the violacein low treatment moves away from the source as time steps increase.

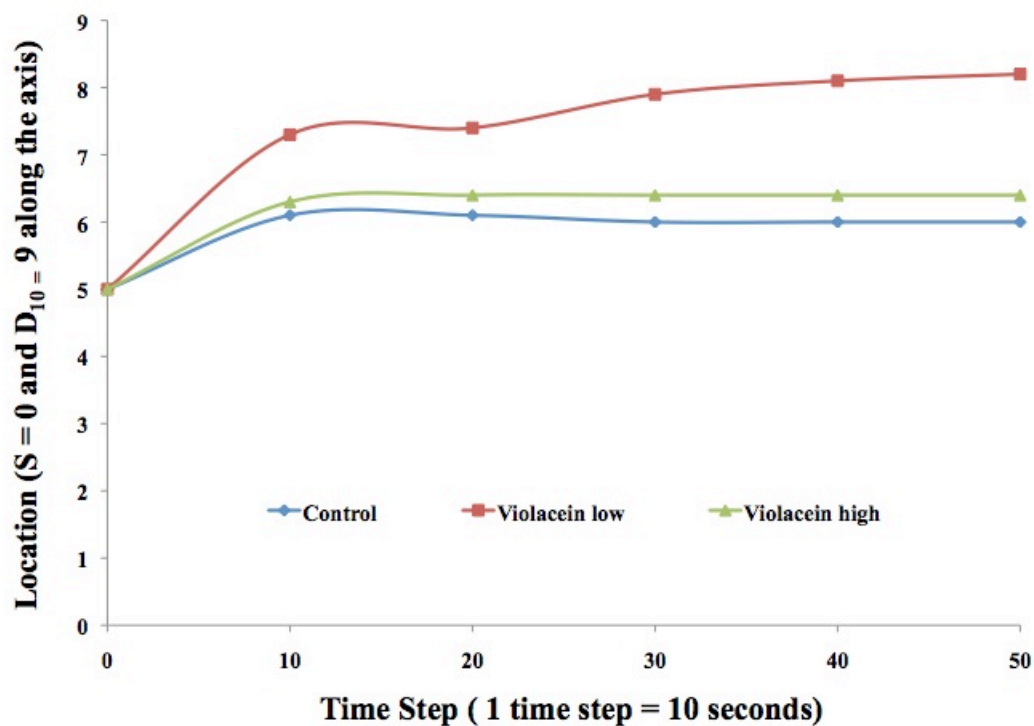


Figure 19. Average number of *B. dendrobatidis* (JEL 423) zoospores at locations in the model at six different time steps (0, 10, 20, 30, 40, 50).

Discussion

Amphibian populations that co-exist with *B. dendrobatidis* do so with sub-lethal infections and several co-existing populations have also been identified with higher proportions of individuals with anti-*B. dendrobatidis* skin bacteria (Lam et al. 2010, Woodhams et al. 2007b). Skin bacteria may drive *B. dendrobatidis* densities down and keep densities below a lethality-threshold resulting in sub-lethal infections. There are three hypothesized mechanisms that skin bacteria may use to drive *B. dendrobatidis* densities down: 1) skin bacteria may reduce the number of available attachment sites on amphibian skin, 2) antifungal metabolites produced by skin bacteria may directly kill *B. dendrobatidis*, and 3) skin bacteria may alter *B. dendrobatidis* zoospore behavior resulting in chemotaxis away from bacterially-produced antifungal metabolites.

The results show that zoospores have chemotactic behavior in the presence of two bacterially-produced metabolites (2,4-DAPG and I3C). Zoospores exhibit chemotaxis in the presence of keratin suggesting that the pathogen has some ability to locate its amphibian host (Moss et al. 2008). I have now also shown that zoospores show a behavioral response to antifungal metabolites that are produced by bacteria isolated from amphibian skin. Zoospores move more frequently away from sources of 2,4-DAPG and I3C.

These results increase support that amphibian skin bacteria play a role in the defense against the amphibian disease chytridiomycosis. More research is needed to determine transmission effects of having different proportions of individuals with anti-*B. dendrobatidis* bacteria. Once an effective proportion of individuals needed to have anti-

B. dendrobatidis to protect a population is found, using bacteria to bio-augment amphibian populations may be a tool for conservational purposes.

B. dendrobatidis Zoospore Movement Patterns

Both isolates of *B. dendrobatidis* zoospores (JEL 310 and JEL 423) had similar behavioral patterns of movement in all treatments. The two most frequent movement patterns were circular and oscillations either inward or outward. Because zoospores moved in this way, the method by which I determined differences in the total zoospore movement either toward or away from the edge of the source (measuring the initial distance a zoospore was from the edge of the agar source and end point distance after ten seconds) may not have been appropriate. Depending on where the zoospore was during its oscillation, total distance traveled might not reflect the general trend moved toward or away from a metabolite. Determining where the zoospore is at its closest point to the edge of the source and at its furthest point at the edge of the source would result in a better overall measurement of total distance a zoospore moves towards or away from a metabolite.

B. dendrobatidis Zoospore Activity and Sampling Time

Two sampling times were chosen to be initial time ($t = 0$ hours) and the time at which 50% of *B. dendrobatidis* (JEL 310) zoospores remained active. The data show that at approximately 6 hours 50% of zoospores from isolate JEL 310 were no longer active. Inactivity suggests that the zoospores were beginning to settle from absorbing their flagella to transition into their sporangium life stage. At 24 hours zoosporangium could

be seen implicating that these zoospores were not dead. These results were different for isolate 423 where approximately 75% of zoospores were still active at 12 hours. Finally, Piotrowski et al. (2004) observed that *B. dendrobatidis* isolate JEL 197 showed approximately 50% of zoospores were active at 18 hours.

The difference in zoospore activity between isolates may be a result genetic variation, however recent studies have found that there is little variation found among isolates (James et al. 2009, Morehouse et al. 2003). Another explanation for the difference in zoospore activity may be the amount of time an isolate is kept in lab after it has been isolated from the wild. Voyles et al. (2009) found that pathogenesis of *B. dendrobatidis* decreased with the amount of time an isolate was kept in the lab. Isolate JEL 310 had been kept in lab approximately a year before it had been used for this experiment. Isolate 423 had only been in the lab for one month before its use and had just recently been cultured from -80°C. Continual making of new weekly stocks of *B. dendrobatidis* may select for zoospores that settle more quickly in the broth culture relative to newly isolated *B. dendrobatidis* from amphibians. Zoospores that settle and encyst quickly in broth cultures may have a higher fecundity in lab than in nature. Zoospores that remain active longer may have a better chance at survival in nature because it allows them more time to search out for susceptible amphibian hosts.

How Amphibian Skin Bacteria May Ameliorate the Effects of Chytridiomycosis

Previous research has shown that amphibians with anti-*B. dendrobatidis* bacteria on their skins suffer less morbidity and mortality when infected with *B. dendrobatidis* than amphibians without anti-*B. dendrobatidis* bacteria (Becker et al. 2009, Harris et al.

2006, Harris et al. 2009a, Harris et al. 2009b). The mechanism(s) of the benefit(s) from having anti-*B. dendrobatidis* bacteria on amphibian skins are not fully known. It is hypothesized that skin bacteria may compete with *B. dendrobatidis* zoospores for attachment sites. As the density of skin bacteria on an amphibian increases, available attachment sites for *B. dendrobatidis* zoospores may decrease and may drive the density of zoospores to stay below the lethal threshold. It is also hypothesized that skin bacteria that produce antifungal compounds that kill *B. dendrobatidis* may also drive the density of zoospores to stay below the lethal threshold by direct mortality. The concentrations of bacterially-produced metabolites are poorly estimated on amphibian skin (Brucker et al. 2008b), but concentrations are hypothesized to be most likely variable and unevenly dispersed on amphibian skin due to the different microenvironments on amphibian skin. It is possible that with large densities of skin bacteria that produce anti-*B. dendrobatidis* metabolites there may be large enough concentrations of the metabolites in many areas dispersed evenly on amphibian skin to cause enough direct mortality of *B. dendrobatidis* to drive the density of zoospores below the lethal threshold. An additional hypothesis may be *B. dendrobatidis* zoospores avoid bacterially-produced metabolites at low concentrations and that the zoospores ability to move away from the metabolites is what drives the zoospore density below lethal threshold.

***B. dendrobatidis* (JEL 310) Chemotaxis in the Presence of Bacterially-Produced Metabolites**

B. dendrobatidis (JEL 310) show chemotaxis in the presence of 2,4-DAPG at low and high concentration and I3C at high concentration. Both of these metabolites have

been shown to be anti-*B. dendrobatidis* in inhibition assays *in vitro*. The metabolite 2,4-DAPG is a known metabolite produced by several *Pseudomonas* species (Weller et al. 2007) and was also identified from *Lysobacter gummosus* isolated from the eastern red-backed salamander, *Plethodon cinereus* (Brucker et al. 2008a, Harris et al. 2006). Many different species of *Pseudomonas* have been isolated from amphibians and have shown antifungal properties, but metabolites have not been identified (Harris et al. 2006, Lam et al. 2010, Lauer et al. 2007, Lauer et al. 2008, Woodhams et al. 2007b). The metabolite I3C was identified from *Janthobacterium lividum* (Brucker et al. 2008b) and this bacteria species has been identified on several amphibians (Harris et al. 2006, Lauer et al. 2007, Lam et al. 2010, Woodhams et al. 2007b). Identification for these metabolites on amphibian skin has not been researched.

Zoospores move more frequently toward a source containing no metabolite than from a source containing either 2,4-DAPG at low and high concentration or I3C at high concentration. Likewise, zoospores also moved more frequently away from a source containing a metabolite than a source containing no metabolite. These data suggest that not only do zoospores have chemotaxis in the presence of these metabolites, but also that overall zoospore movement is away from the metabolites. Additional observations indicated that zoospores were concentrated at the farthest distance from the metabolite source, which supports this conclusion.

It is hypothesized that metabolites would form a concentration gradient from the source containing the metabolite and the furthest location away from the metabolite in solution. However, location had no effect on zoospore movement either towards the metabolite or away from the metabolite for the control, 2,4-DAPG low and I3C high

treatments. This may be due to the small volume of Provosoli that each chamber held. In the future concentration gradients should be measured. If the chambers do not represent a concentration gradient then it is likely that if the chambers were made to be longer and measurements taken further from the source then the effect of location may have been seen. However, casual observations showed that zoospore density was highest at the far edge of the chamber away from the metabolite. In addition, it is likely that a gradient was present at $t = 0$ and behavior was the same six hours later. This observation also suggests a gradient established. These observations, along with the bulk of the chemotaxis data, suggest that a concentration gradient was established.

The metabolite 2,4-DAPG at high concentration did show an effect of location on zoospore movement both towards the metabolite and away from the metabolite. Zoospores moved towards 2,4-DAPG high more frequently further away from the metabolite and moved less frequently away from the metabolite as distance increased. It is possible that at the high concentration of 2,4-DAPG there may have been deleterious effects on the zoospores that cause their movement to be erratic.

B. dendrobatidis (JEL 423) Showed No Chemotaxis in the Presence of Bacterially-Produced Violacein

B. dendrobatidis isolate JEL 423 showed no chemotaxis in the presence of violacein at either low or high concentration. There are several explanations for this that need further investigation. One explanation may be that there is variation between the two isolates (JEL 310 and 423) either due to genetic variation or due to variation in their pathogenesis and behavior based on number of generations in the laboratory. If JEL 423

has a higher pathogenesis than JEL 310 it could explain why JEL 423 showed no chemotaxis in the presence of violacein. Behavior can be a part of determining the pathogenesis for an organism. Perhaps one factor making isolates highly pathogenic is due to the isolates ability to withstand bacterially-produced metabolites such as violacein. Another explanation for why JEL 423 showed no chemotaxis in the presence of violacein may be due to the metabolites insolubility in Provosoli medium. Repeating this experiment again with 1% dimethyl sulfoxide (DMSO) in addition to the Provosoli medium may allow for the metabolite to form a concentration gradient and may change the chemotaxis of zoospores in the presence of violacein. However, this brings about the question of how likely is this in nature? If violacein is insoluble in Provosoli medium than does it form a concentration gradient in the mucus layer it is found it? One possibility is that *Janthinobacterium lividum* that produces violacein (Brucker et al. 2008b) also secretes a molecule that makes the violacein soluble. More research is necessary to fully understand the mechanism(s) that insoluble bacterially-produced metabolites use to ameliorate chytridiomycosis.

B. dendrobatidis (JEL 310) Zoospore Activity in the Presence of Bacterially-Produced Metabolites

There were more active zoospores at $t = 0$ hours than at $t = 6$ hours within the control and all three of the treatments (2,4-DAPG low, 2,4-DAPG high, and I3C high). This is not surprising considering that 50% of the zoospores (JEL 310) are no longer active at 6 hours and this likely contributes to the effect of time. Location had no effect

on active zoospores for within all treatments with the exception of 2,4-DAPG at high concentration.

The control and 2,4-DAPG treatments had similar numbers of active zoospores than the 2,4-DAPG high treatment. This result may have been due to direct mortality of a proportion of zoospores exposed to a high concentration of 2,4-DAPG. There was no effect of treatment on the number of active zoospores at two locations sampled between the control and I3C at high concentration.

B. dendrobatidis (JEL 423) Zoospore Activity in the Presence of Violacein

There was no significant effect of treatment on *B. dendrobatidis* (JEL 423) zoospore activity between the control and both violacein at low and high concentrations. Explanations for this are similar to the explanations to explain why there were no effects of violacein on zoospore movement towards or away from the source.

B. dendrobatidis (JEL 310) Zoospores Take Longer to Reach the Source and Are Less Likely to Distribute on The Source in the Presence of Bacterially-Produced Metabolites

Using parameters estimated from data collected on zoospore movement, it was observed that when a single zoospore was released from the middle of the model (D₅) it reached the source more quickly in the control than the three treatments (2,4-DAPG low, 2,4-DAPG high, and I3C). This suggests that if zoospores were released into the environment they would reach amphibians without bacterially-produced metabolites more quickly than amphibians with bacterially-produced metabolites.

The model to illustrate the transmission of 200 zoospores that are released in the furthest location of the model (D₉) shows that as time increases zoospores move in locations further away from the source in all experimental treatments. Zoospores in the control treatment move towards locations closer to the source as time increases. This suggests that if zoospores are released into the environment that amphibians that have bacterially-produced metabolites on their skins would have a lower proportion of zoospores that infect the amphibian than those that do not have bacterially-produced metabolites on their skins.

This model also illustrates that zoospores released from sporangia located on frog skin that also have antifungal metabolites will leave the frog skin and this may drive zoospore density to remain below the lethal-threshold and also increase transmission to susceptible amphibians. In the presence of no metabolite, zoospores released from sporangia located on amphibian skin are more likely to remain on frog skin and re-infecting the amphibian. This could drive exponential growth of zoospores on amphibian skin and quickly surpass lethal-threshold.

B. dendrobatidis (JEL 423) Zoospores Are Equally Likely to Reach the Source and Are Less Likely to Distribute on the Source in the Presence of Violacein at Low Concentrations

The model predicts that more zoospores will take longer to reach the source in the violacein low treatment compared to the control and violacein high treatment. This suggests that if an amphibian has low concentrations of violacein on its skin, zoospores will take longer to reach the amphibian.

The model to illustrate the transmission of 200 zoospores that are released in the middle of the model (D_5) shows that as time increases zoospores move in locations further away from the source in the violacein low treatment. Zoospores in the control and violacein high treatments first move away from the source and then gradually move closer towards the source. This suggests that if zoospores are released into the environment that amphibians that have violacein in low concentrations on their skins would have a lower proportion of zoospores that infect the amphibian than those that do not have violacein or have violacein at high concentrations on their skins.

Conclusion

The results of this study show that *B. dendrobatidis* zoospores show chemotaxis in the presence of two bacterially-produced metabolites and that zoospores are less likely to distribute onto a source containing metabolites than a source that does not have metabolites. Chemotaxis of zoospores in the presence of metabolites may be a mechanism driving and maintaining zoospore density on frog skin to stay below the lethal threshold. Skin bacteria that produce anti-*B. dendrobatidis* metabolites may ameliorate the effects of chytridiomycosis on amphibians that host them. These amphibians may also co-exist with *B. dendrobatidis* even if sporangia are found on their skins if sporangia are found at very low numbers.

Understanding all of the components of the amphibian innate immune system and defenses and how the interactions between the components protect amphibians from diseases such as chytridiomycosis needs more research. Future research should focus on synergistic or antagonistic relationships between amphibian skin bacteria and amphibian

produced AMPs. Identifying population density and distributions of skin bacteria on amphibian skin in different microenvironments is needed to infer how effective bacterially defenses are. There is need to understand how zoospores move in pond water compared to the mucus on the amphibian skin. Also needed is a determination of the most effective concentration of metabolites needed to produce the maximal effects of bio-augmentation. Results of I3C and violacein at high concentration suggest that these metabolites may be more effective at lower concentrations to encourage the smallest proportion of zoospores to disperse to frog skin. For conservation purposes, bio-augmentation of amphibian skin with too much bacteria may be costly for the host to maintain or may cause disease as many commensal bacteria are shown to do at high densities. However, bio-augmentation of amphibian skin bacteria with an appropriate number of bacteria may have the desired effects of ameliorating chytridiomycosis without any cost to the amphibian.

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